



# PROTEIN and AMINO ACID NUTRITION

*Edited by*

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*Greenwich, Connecticut*

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## Preface

During the past few years there have been many requests that the monograph *Protein and Amino Acid Requirements of Mammals*\* be brought up to date and expanded in scope. Moreover fellow investigators have indicated that there is need for a collection in one volume of rather detailed presentations describing the current state of knowledge concerning this aspect of nutrition. This volume aims to fill that need.

The unprecedented research activity in this field and in related areas during the past decade has resulted in a gradually increasing limitation of space in the periodical literature which can be allotted to review, reflections and speculations on the broad significance of experimental findings. These publication restrictions tend to retard the continuity of scientific thought and progress. One solution to the problem appears to be the use of media which can provide qualified investigators ample space and freedom to express themselves fully in the area of their major cognizance.

Although all segments of human communication are currently beset by difficulties of orderly and accurate transmission of facts, the field of nutrition falls prey to some unique hazards. The most serious of these results from the vast number of reports dealing with nutritional matters which appear almost daily in the lay literature. No one will deny the value of public education in this most vital of subjects. Competition for space and readers, however, tends to lead to the dramatization of certain scientific reports at the expense of accuracy. Although such accounts of scientific observations in the public press can be condoned somewhat by the expediency of the circumstances, the increasing appearance of these articles in the professional literature is to be deplored and constitutes a disservice to the science of nutrition, particularly when the editorial responsibility is cloaked with authoritarian anonymity.

The Editor wishes to acknowledge with deep appreciation and thanks the generous collaboration of the individual contributors which has made this volume possible. He feels particularly indebted to his colleague and mentor for more than a decade, Dr. Reginald A. Higgons, for his assistance in many of the editorial and reviewing tasks connected with this publication. Acknowledgment is also due Mrs. Muriel E. Rosenquest and Miss Louise A. Orto for their valuable help and industry in proof

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ANTHONY A ALBANESE

*Greenwich, Connecticut*  
*June, 1959*

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## CHAPTER I

# Introduction and Perspectives

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Perusal of the contents of the present volume will reveal that the past decade has witnessed considerable progress in our understanding of the nutrition of proteins and amino acids. It will be noted in particular that the development and application of new procedures has not only greatly expanded the field of inquiry in experimental animals but also has made possible the conduct of exact nutritional and biochemical measurements in human subjects of all ages and under a variety of physiological and pathological conditions. Under the continuing assault of micro and radiochemistry we have learned that the quantitative and qualitative nutrient needs of mammals are subject to significant species (Mitchell 1959) and individual (Williams 1959) differences and that the need for a specific nutrient in any single species is defined by an almost infinite number of variables and that the protein value of foods is susceptible to an equally large number of vicissitudes. This development of minutiae however does not relegate to the discard the general metabolic laws of nutrient needs and waste per unit of surface area developed so ably in the first quarter of this century (Brody 1945). Rather it points to the fact that maintenance of a necessary metabolic rate and function per unit of surface area varies for specific nutrients from individual to individual and from species to species. This should come as no surprise if we consider that no two energy converters organic or inorganic will function with identical efficiency.

In living organisms these differences are ultimately traceable to variations in available enzymes or other biocatalysts (Martin 1958). This aspect of human chemistry is not new. It received attention and documentation by Garrod as long ago as 1923 under the title "Inborn Errors of Metabolism." Today because of the wonders of chromatography we have micro tests for almost every conceivable organic constituent. Thus we are able not only to ascertain the presence of classic metabolic aberrations (alkaptonuria, phenylketonuria) with greater frequency but also to discover new and heretofore unsuspected biochemical lesions (Dent 1947 Harris 1955). Such lesions have been produced artificially by deprivation of a single amino acid (Albanese 1952 Hall *et al* 1948). Administration of antimetabolites has been shown to result in similar



pathological lesions (Woolley, 1952) and congenital malformations (Warkany, 1958)

The past decade has also shown that as the periphery of specific areas of study is expanded there occurs a rapid fusion and eventual disappearance of boundaries. Thus, we find that both the catabolic and anabolic phases of the metabolism of the amino acids are inextricably related to the metabolism of fats and carbohydrates (Swanson 1959), and they cannot be studied or discussed without some reference to nutrition in general. This becomes quickly apparent from a consideration of the currently known metabolic interrelationships of the amino acids (Fig 1). Continued study of the nutritional implications of these metabolic linkages may be expected in the proximate future to yield more exact knowledge on specific protein and amino acid needs. Accordingly it seems worth while here to delineate briefly the current state of development of some of these relationships.

## I VITAMINS AND AMINO ACID METABOLISM

From the metabolic diagram (Fig 1) it is at once apparent that the biochemistry of amino acids is integrated with the over all metabolism and all of the vitamins that are dietary essentials are involved in normal metabolism. In general, the role of the vitamins is that of serving essential parts of the molecular structure of the enzyme systems that constitute the metabolic machinery of the cells. Many gross effects, such as lack of growth, are common to a number of vitamin deficiencies as well as some amino acid deficiencies. It has been shown (Kinney and Folts 1958) that some pathological lesions produced in experimental animals by amino acid deficiencies are indistinguishable from those caused by vitamin deficiencies. Goldsmith (1956) has skillfully clarified the interdependence of the intermediary metabolism of niacin and tryptophan and the effect of this biochemical circumstance on the nutritional needs of these two dietary essentials.

Specific evidences of even more subtle interrelationships are now on hand. Some six years ago, some babies receiving a proprietary milk formula began to show excessive irritability and in many instances convulsions (Bessey 1957). On the basis of similar symptoms in experimental animals and a consideration of the way in which the product was manufactured, a deficiency of vitamin B<sub>6</sub> was suspected. This suspicion was soon confirmed by the favorable response of the infants to formulas with an increased vitamin B<sub>6</sub> content.

Biochemical studies have revealed that animals deficient in vitamin B<sub>6</sub> excrete xanthurenic acid in the urine and this excretion is also increased after the administration of a test dose of tryptophan (Robinson 1951).

In the absence of vitamin B<sub>6</sub> xanthurenic acid now known to be an abnormal product of tryptophan metabolism is formed in increasing quantities. In babies with convulsions xanthurenic acid disappeared after B<sub>6</sub> therapy was instituted. In all cases except two the level of

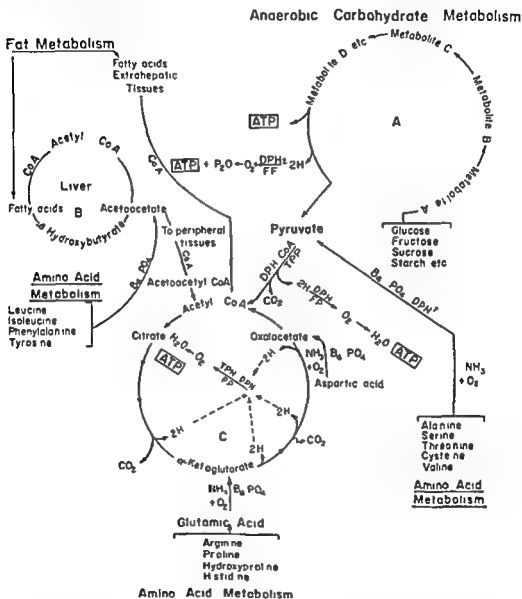


FIG. 1 Metabolic integration. ATP = Adenosine triphosphate. DPN<sup>+</sup> = diphosphopyridine nucleotide (niacin in structure). TPP = triphosphopyridine nucleotide (niacin in structure). TPP = thiamine pyrophosphate (thiamine in structure). FP = flavoprotein (riboflavin in structure). CoA = coenzyme A (pantoic acid in structure). B<sub>6</sub> PO<sub>4</sub> (vitamin B<sub>6</sub> in structure). From Bessey (1957).

vitamin B<sub>6</sub> required to eliminate xanthurenic acid excretion was considerably above that required to eliminate convulsions. Apparently the amount of vitamin B<sub>6</sub> required for normal metabolism of tryptophan is greater than the amount required for minimum function of some as yet unknown biochemical properties of nervous tissue. These facts now form the basis for a test of vitamin B<sub>6</sub> status in infants.

Evidences, although not as clear cut as the foregoing are on hand regarding the relationship of most known vitamins to protein and amino acid nutrition. The important role of vitamin B<sub>12</sub> in the biosynthesis of proteins is now generally acknowledged. A deficiency of vitamin B<sub>12</sub> reduces nucleic acid synthesis and thus causes a decrease in cell division. This has been demonstrated in human bone marrow cells from pernicious anemia patients (Glazer *et al*, 1954). Rats receiving B<sub>12</sub> will grow on diets deficient in choline and methionine, but containing homocystine. This suggests that the vitamin has a function in the metabolism of labile methyl groups (Bennett 1950). In hyperthyroid rats B<sub>12</sub> has a protein sparing action. This does not prevent weight loss, however, for protein is spared at the expense of other body constituents (Rupp *et al*, 1951). This vitamin also permits utilization of nitrogen in animals fed a diet high in soybean protein (Catron *et al*, 1952, Hsu *et al* 1953). Since the methionine content of soybean protein is low, this effect is most likely related to the methionine sparing activity of B<sub>12</sub> rather than to a direct effect on protein synthesis.

Less clear are the relationships of vitamins C, A, and E to protein and amino acid nutrition. The lesions which deficiencies of these vitamins induce in both experimental animals and man leave no doubt that they participate in the synthesis of many specialized tissues. For example one of the most important roles of ascorbic acid involves the formation of collagen in teeth, bone, cartilage, connective tissue and skin. It promotes normal development of the teeth including both pulp and dentine (Fish and Harris 1935) but apparently does not influence the occurrence of dental caries in man. Ascorbic acid has also been reported to be essential for regeneration of damaged nerve tissue (Hines *et al* 1944). Diets high in protein increase the excretion of ascorbic acid.

Although the function of vitamin A in the chemistry of the visual purple is now clearly understood, its function in maintaining the integrity of epithelial cells and as a stimulus for new cell growth remains obscure. Vitamin E deficiency leads to progressive muscular dystrophy in experimental animals, but vitamin E has no beneficial effect, even in massive doses, on muscular dystrophy occurring in man. The investigative opportunities in this area seem endless.

## II EFFECT OF SOME THERAPEUTIC AGENTS ON PROTEIN AND AMINO ACID NUTRITION

Although the number and diversity of pharmacological agents having rather well defined effects on metabolism continues to grow, very little effort has been made to date to correlate the metabolic or nutritional disturbances with either pharmacological activity or chemical structure of these agents. The ever increasing interest in nutrition will of course result in a growing need to assay new therapeutic agents not only for their pharmacological properties but also for their nutritional characteristics.

### A ANTIBIOTICS

The subject of antibiotics in nutrition was admirably reviewed by Jukes (1955). It is clear and well established from available evidence that an apparent improvement in the nutritional status of animals and humans may often be produced by adding small quantities of certain antibiotics to the diet. The mechanism by which their effects on nutrition are produced appears to be secondary to their antibacterial action. Stokstad (1955) has discussed the effect of antibiotics on vitamin requirements. He notes that while a vitamin sparing action can explain in part the growth response on diets marginal in vitamins, it does not account for the growth observed in nutritionally complete rations. The vitamin sparing effect of the antibiotics has been observed to occur for both water soluble and fat soluble vitamins. A thorough consideration of the data leads Coates and Kon (1955) to believe that although the growth promoting effect of antibiotics depends to a large extent on their antibacterial properties, their direct effect on certain metabolic processes cannot be overlooked. Hence it is conceivable that given in the diet they exert a small but definite pharmacological action which contributes to their beneficial effects on growth.

There are currently growing indications that the combined administration of antibiotics and amino acids may help resolve some nutritional problems in technologically underdeveloped areas of the world. Because of the impracticability of supplying to many children living in tropical countries diets rich in high quality proteins, it often happens that their growth is slow and they become stunted. Since antibiotics and other substances such as lysine have growth stimulation effects on children in temperate zones, Loughlin and associates (1957, 1958) decided to try these agents in chronically malnourished tropical children. Sixty-four children (6-16 years) at a rural school in Haiti were selected for study. After their conditions had been analyzed they were fed under controlled study conditions; the children were separated into four matched groups.

In three, supplements to their diets were used in the form of 10 mg and 50 mg of oxytetracycline and 1 gm of lysine, respectively. The fourth group received placebo. The study was run on a double blind basis, and lasted for 6 months. On analysis it was found that the group that received 50 mg of oxytetracycline daily manifested the best growths. In growth factors the group receiving lysine came out second. There were no toxic or untoward effects noted as the result of this long range administration of oxytetracycline or lysine.

A critical evaluation of the role of nutrition in the prophylaxis and treatment of disease reveals many other areas in which proteins and antibiotics may jointly serve to improve over all metabolism and reduce the incidence of infections (Hulpern, 1955).

### B STEROIDS

The rapidly increasing availability and usage of new synthetic catabolic and anabolic steroids provides a limitless opportunity for study of basic metabolic problems of proteins and amino acids (Luetscher and Lieberman, 1958). Some of these possibilities are touched upon in other sections of this volume. It is clear from the evidence on hand that control of the nitrogen depletion effects of the corticoids can be achieved in part by protein supplementation of the dietary and in some instances, by administration of small quantities of some of the essential amino acids. Similarly it appears that effects of some anabolic steroids may be enhanced by amino acid fortification of the dietary. Closer scrutiny of this nutritional synergism may lead to attainment of maximal anabolism at very small dosage levels of these steroids and thereby a reduction in the incidence of virilism and other untoward effects.

### C HYPOGLYCEMIC AGENTS

The fortuitous observation of Janbon *et al* (1942) that the newer sulfonamides produced a disorder very similar to hypoglycemia led to Loubatiere's studies (1957) on the mechanism of action of the sulfonylureas in diabetes and eventually to an evaluation of these substances for the control of diabetes (Dolger, 1957). A pilot study on two diabetics given rather large doses of tolbutamide for about 2 weeks showed no changes in respiratory quotient in either subject, and a slightly negative nitrogen balance in one (Goetz, 1957). McGavack and associates (1957) observed that whereas 1 gm daily of carbutamide did not influence the uptake of radioiodine, 2 gm daily of carbutamide progressively depressed  $I^{131}$  uptake to 56% of the control value at the end of the ninth week of treatment.

Numerous reports have appeared implicating salicylates in carbohy

hydrate metabolism particularly the reported ability of aspirin to lower blood sugar levels in diabetics (Smith 1953). A definite hypoglycemic effect of salicylates was demonstrated in rheumatic fever patients by Albani and co-workers (1955). Results of the study which involved ten subjects (5-18 years) indicated that the magnitude of fasting hypoglycemia produced by aspirin bears a direct relationship to the duration and dosage of therapy and the age and weight of the patient. Subsequent studies by this group (Albani 1959) have disclosed that the hypoglycemia of prolonged and massive salicylate therapy is sometimes accompanied by a persistent decrease in fasting plasma amino nitrogen levels—a sensitive criterion of protein nutrition.

A further and heretofore unsuspected relationship of amino acid and carbohydrate metabolism has been described by Seltzer and Smith (1958). These investigators reported that in normal subjects and in mild or severe diabetics tolbutamide (Orinase) and indole 3 acetic acid—a physiological end product of tryptophan metabolism exerted identical effects on blood glucose and plasma insulin activity in the same individuals.

#### D. PHENOTROPIC DRUGS

The pharmacology of this rapidly growing family of substances was thoroughly reviewed in 1957 by a very able conference group (Kety 1957). The psychotherapeutic activity of some of the agents in common use was reported to correlate well with their inhibition of oxidative phosphorylations (Fig. 1) and/or cytochrome oxidase. Preliminary observations (Abood and Romanchek 1957) have disclosed that many indole substances and derivatives of the urine of phenylketonurics are also formidable inhibitors of cellular oxidations and phosphorylations. Recently these aromatic substances which are metabolically derived from tyrosine and tryptophan were suspected of a possible role in the biogenesis of schizophrenia producing compounds.

It is interesting to note in this connection that dietary deficits of niacin which is also derived biosynthetically from tryptophan have been implicated in the etiology of various psychiatric symptoms which were relieved by niacin administration (Jolliffe *et al* 1940). Isoniazid an isomer of nicotinic acid has been observed to produce marked euphoria early in experimental trials in tuberculosis (Bennett *et al* 1954).

From the foregoing it is apparent that investigations on the biochemical properties of proteins and amino acids may have ramifications in many areas far removed from their nutritive functions. Also from the preceding as well as the pages which follow it will become abundantly

clear that future progress of the science of nutrition will depend more and more on the mutual understanding and collaborative efforts of investigators working at all levels of biochemical organization—from two carbon molecules to the whole man

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## CHAPTER 2

# Some Species and Age Differences in Amino Acid Requirements

H. H. MITCHELL

*Division of Animal Nutrition, University of Illinois, Urbana, Illinois*

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## I INTRODUCTION

The unique functions in the animal body served by the amino acids resulting from protein digestion are all anabolic in character. They relate to the replacement of essential tissue constituents that have been degraded in catabolic reactions or to the formation of new tissue constituents in growth. In the rapidly growing animal, the latter functions dominate the body's requirements for amino acids. In the mature animal the replacement functions may dominate the amino acid require-



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## I INTRODUCTION

The unique functions in the animal body served by the amino acids resulting from protein digestion are all anabolic in character. They relate to the replacement of essential tissue constituents that have been degraded in catabolic reactions, or to the formation of new tissue constituents in growth. In the rapidly growing animal the latter function dominates the body's requirements for amino acids. In the mature animal the replacement function may dominate the amino acid require-

ments but the growth functions still persist, since some tissues continue to grow throughout life (Mitchell 1949) Among adult animals of different species the relative importance of the growth functions in determining amino acid requirements will depend mainly on the rate of growth of the epidermal structures, such as hair, nails, and claws

The different types of anabolic reactions occurring in the growing and in the adult organism with reference to the disposal of dietary amino acids lead inevitably to the conclusion that the amino acid requirements of infancy and adolescence, on the one hand and of maturity on the other hand, are different either in the assortment of amino acids that are required preformed in the diet, or in the relative amounts that are needed or both. Species differences in amino acid requirements during growth would be expected not so much on the basis of differences in the nature of the anabolic reactions as on the basis of differences in the rate of growth of new tissue in comparison with the weight of tissue to be maintained that is, the percentage rate of growth. The higher the percentage growth rate, the greater the extent to which the growth of new tissue dominates the total amino acid requirements. The rat, growing at a rate of 3% (or more) of its body weight daily, would need amino acids more in proportion to the needs for the formation of new tissues. The amino acid requirements of the human child growing at a rate of some 0.03% of its body weight daily would be dominated more by the needs for amino acids to replace endogenous losses. In the former case the daily requirement of protein, following the point of inflection in the growth curve, would be expected to decrease in absolute amount as the daily growth increments decrease. In the latter case, the amount of protein required per day may be expected to increase as the body weight increases, but at a slower rate.

The amino acids needed for growth are needed mainly for the synthesis of the protein molecules entering into the structure of protoplasm. For this function the presence in the tissues of all of the amino acids that the body cannot manufacture itself from dietary constituents is required simultaneously or nearly so. The absence of any one will block the synthetic processes. The amino acids needed for maintenance are needed mainly for the formation of creatine, carnosine, glutathione, ergothione, thyroxine, adrenaline, and other nitrogenous tissue constituents or tissue products destroyed in metabolism. For these replacement functions the assortment of amino acids needed is simple and will vary from one type of synthetic reaction to the other. The absence from the diet of any one essential amino acid will block one or more of these anabolic reactions but not all. Hence a dietary protein like gelatin or zein entirely deficient in one or more of the amino acids that the body

cannot manufacture itself may be partially utilized in maintenance as McCollum and Steenbock (1912) showed many years ago in metabolism experiments on growing pigs. For growth gelatin possesses a biological value of zero, since no growth will occur with gelatin as the sole source of amino acids. But for maintenance it possesses a biological value of about 25 (Arnold and Schach 1952, Block and Mitchell 1946, Rhode *et al.* 1949) indicating that many of the replacement reactions of maintenance do not need the amino acid entirely lacking from the gelatin molecule, i.e., tryptophan. Maintenance will also involve some protein synthesis for the growth of epidermal structures for the replacement of red blood cells destroyed during metabolism and probably for many other purposes although the net aggregate in terms of nitrogen may not be large in proportion to the total replacement aggregate.

An important recent development in amino acid nutrition is the demonstration that the nonessential amino acids can be synthesized in the body of the monogastric animal from glutamic acid and the unnatural forms of the essential amino acids (Anderson and Nasset 1945) from ammonium citrate (Lardy and Feldott 1949) and from glycine and urea (Rose *et al.* 1949). The *ad libitum* feeding technique employed in the latter two reports detracts to some extent from the conclusiveness which the interpretation of the nitrogen balance data might otherwise possess. The utilization of ammonia for amino acid synthesis (Rittenberg *et al.* 1939, Schoenheimer *et al.* 1939, Sprinson and Rittenberg 1949a,b) but not that of urea (Bloch 1946 and Bloch *et al.* 1941) has been confirmed by the use of appropriate dietary supplements labeled with the  $N^{15}$  isotope. A later report by Rose and Dekker (1956) presented evidence that "the nitrogen of urea can be utilized for the synthesis of the nonessential amino acids when the latter are excluded from the food and when no other source of nitrogen is available for the purposes in question." The possible intervention of intestinal bacteria in the mechanism of urea utilization is recognized.

## II AN EXPERIMENTAL STUDY

The experiments that will be reported in this section were designed to test the statements just made concerning the differences in amino acid requirements that may be expected to accompany differences in age and in species among animals. They were carried out upon young and upon mature rats not with different mixtures of amino acids but with different proteins known to differ in amino acid content and in nutritive quality. The results obtained will be compared with those secured with adult human subjects subsisting on the same protein sources.

Most of the data on human subjects will be taken from an article



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Most of the data on human subjects will be taken from an article

published in 1948 from Murlin's laboratory at the University of Rochester (Hawley *et al.*, 1948). Many of the data on growing and adult rats have been taken from a paper by Mitchell and Beadles (1950). Both series of experiments were part of a cooperative study of protein utilization sponsored by the Bureau of Biological Research of Rutgers University. The proteins, or protein foods, used by all cooperating laboratories were the same consisting of egg albumin, desiccated and defatted whole egg dried and defatted beef muscle, wheat gluten casein and peanut flour.

### A EXPERIMENTAL METHODS

The experiments both those at the University of Illinois and those at the University of Rochester, were carried out in accordance with the nitrogen balance method, yielding coefficients of true digestibility and biological values. In the work on growing rats and in Murlin's work on adult men and women, these measures of protein utilization in digestion and in metabolism were computed by the Thomas method or a modification of this method to adapt it to the growing animal. In the work on adult rats, the calculations were made on the basis of data secured with three dietary levels of each test protein; the biological values were computed from the slope of the line describing the relationship of absorbed nitrogen and of nitrogen balance at levels of intake insufficient to support nitrogen equilibrium or to induce any considerable nitrogen retention (Bricker and Mitchell, 1947), a modification of a method introduced by Melnick and Cowgill (1937). The data were expressed per calorie of basal metabolism and were pooled together for each protein in computing regression equations. The standard errors of the slopes of the regression lines were computed by a method described by Rider (1939).

### B EXPERIMENTAL RESULTS

In Table I the biological values of the nitrogen in the six protein sources for growing rats for mature rats and for adult humans are assembled for comparison. The differences in digestibility not given in the table, are not great with one or two exceptions and their discussion does not seem to be a profitable undertaking since the factors determining the completeness of protein digestion are so little understood.

### C INTERPRETATIONS

The differences in biological value however may be correlated to some extent with the amino acid composition of the proteins. Beef muscle casein and peanut flour are deficient in cystine or methionine or

both for the growing rat while wheat gluten is markedly deficient in lysine (Block and Mitchell 1946)

From the table it will be evident that for those proteins deficient in cystine methionine i.e., beef casein and peanut the biological values are lower for the adult rat than for the growing rat and, with the exception of beef they are lower than for the adult human. The cystine methionine deficiency of beef muscle is comparatively small. On the other hand for wheat gluten deficient in lysine the biological value for the adult rat is considerably higher than for the growing rat or for the adult human.

TABLE I

THE BIOLOGICAL VALUE OF THE TEST PROTEINS BY GROWING RATS MATURE RATS AND ADULT MEN

Protein	Biological value		
	Growing rats	Mature rats	Mature human
Egg albumin	97	94	91
Whole egg (commercial)	87	82	94
Beef muscle	76	69	67
Wheat gluten	40	63	42
Casein	69	51	56
Peanut flour	54	46	56

With regard to egg albumin and the proteins of whole egg no such comparisons can be made among growing rats mature rats and mature humans because no conclusive information is available concerning their limiting amino acids. The estimations of Mitchell and Block (1946) based upon the comparative amino acid analyses of whole egg proteins and of egg albumin would lead one to suspect a small lysine deficiency in the latter protein.

#### D. A TEST OF THE AMINO ACID ADEQUACY OF WHOLE EGG PROTEINS

The nutritive quality of whole egg proteins and of egg albumin is so high that its limitation by amino acid deficiencies if such exist would be expected to be slight. If whole egg proteins are a perfect protein mixture for the growing rat in the limited sense that they contain the essential amino acids in the same proportions that exist among the requirements for these amino acids then supplementation of whole egg proteins with individual essential amino acids should not improve their growth promoting value. In testing this proposition experimentally, a whole egg sample prepared in the laboratory by fat extraction and dehydration at low temperatures was used. Samples prepared by this method have yielded biological values for the growing rat of 94 to 96

regression equations of nitrogen balance on nitrogen absorbed, both being expressed per basal calorie, by solving for absorbed nitrogen in take when nitrogen balance is zero. The values for the adult human were computed in an analogous fashion from the data of Hawley and associates (1948), supplemented by further necessary information kindly provided by Dr J R Murlin. It should be noted that the loss of nitrogen in the sweat of human subjects observed under sweating conditions is not ordinarily measured in nitrogen metabolism studies. On low protein diets it may introduce an appreciable but indeterminate error in the computation of nitrogen balances and biological values (Mitchell *et al*, 1949).

The protein values expressed as milligrams of absorbed nitrogen per basal calorie required for nitrogen equilibrium, for mature rats and for mature humans, are listed in Table III. This table contains also data from some other sources than the two experiments heretofore considered.

TABLE III

THE REQUIREMENTS OF ABSORBED NITROGEN FROM DIFFERENT SOURCES FOR NITROGEN EQUILIBRIUM BY ADULT RATS AND ADULT HUMANS

Protein source	Absorbed N required for N equilibrium	
	Adult rat	Adult human <sup>a</sup>
(mg N per basal cal)		
I Proteins of unknown deficiencies		
Whole egg laboratory prepn <sup>b</sup>	2 16	—
Whole egg commercial	2 63	2 08
Egg albumin	2 30	2 14
II Proteins deficient in cystine methionine		
Beef muscle	3 11	2 93
Casein	4 24	3 31
Peanut flour	4 70	3 51
Soy flour	5 48	2 65
Milk	3 18	2 59
III Proteins deficient in lysine		
Wheat gluten	3 20	4 67
Wheat flour	3 32 <sup>c</sup>	4 61 <sup>c</sup>

<sup>a</sup> Unless otherwise indicated these data were derived from the values reported by Hawley *et al* (1948)

<sup>b</sup> This figure is the average excretion of nitrogen on a nitrogen free diet of the adult rats in this experiment. Slightly deficient in lysine

<sup>c</sup> The rat data are taken from Bricker and Mitchell (1947) the data on humans from Bricker *et al* (1945)

<sup>d</sup> Computed from a biological value of 65 (Mitchell 1947) and the value of 2 16 mg of nitrogen excreted per basal calorie on a nitrogen free diet

The values reveal again the more intense requirement of the adult human than of the adult rat for lysine and the less intense requirement for methionine cystine. If a protein is low in lysine more of it is required per basal calorie by man than by the rat, but if it is low in methionine cystine the reverse is true.

These results are in harmony with other experiments having a similar import. For example Mueller and Cox (1947) showed that casein and lactalbumin are equally effective in maintaining nitrogen balance in adult man. For the growing rat the biological value of casein is much less than that for lactalbumin 69 vs 84 (Kik 1938), but after cystine supplementation they are practically the same 83 vs 85. No similar comparison of casein and lactalbumin for the mature rat has been found in the literature.

Mitchell (1947) observed no supplementing effect of lysine on the metabolic utilization of the proteins of white flour in the adult rat from which he concluded on the basis of this and other pertinent evidence that "lysine is entirely dispensable in adult rodent nutrition or is required in inconspicuous proportions for the maintenance of nitrogen equilibrium" (see also Nasset 1946) in the sexually inactive rat. The latter reservation is inserted because of the finding of Pearson (1937) that a lysine deficient diet induces cessation of estrus cycles in the adult female rat. The latter alternative in the quotation is not inconsistent with the later work of Wissler and associates (1948) in Cannon's laboratory. Contrast with these experiments of Mitchell (1947) and especially the earlier ones by Osborne and Mendel cited by Mitchell the one reported by Bricker *et al* (1945) in which on a white flour diet an adult college woman was in marked negative nitrogen balance. When the diet was supplemented with lysine the urinary nitrogen excretion per day dropped immediately by an amount equal to 3.5 times the nitrogen contained in the added lysine and her nitrogen balance became positive. The need for lysine by the adult human had been previously shown by Rose (1944) and by Albansen and co workers (1941). Of particular interest in this connection are the experiments of Hoffman and McNeil (1949) showing with 10 hospital patients that the nitrogen balance index of Allison *et al* (1947) for the nitrogen of wheat gluten is raised from 0.62 to 0.76 by lysine supplementation.

### III KERATIN SYNTHESIS IN PROTEIN NUTRITION

The relationships discussed above indicate that the cystine methionine requirement is relatively more intense for the adult rat than for either the growing rat or for the adult human. On the other hand the lysine requirement seems to be much less prominent among the amino acid

requirements of the adult rat than among those of the growing rat or the adult human. How may these age and species differences be best explained?

The most probable explanation rests on the high cystine and low lysine content of hair and other keratins (Block and Bolling 1945) and on the probability that the amino acid requirements of the adult rat are dominated by the requirement for hair growth. The scleroproteins of hair contain 14 to 16 gm of cystine and less than 1 gm of methionine for 16 gm of nitrogen and from 2 to 3.8 gm of lysine. The figures for lysine are similar to those reported for wheat gluten (2.0 gm per 16 gm of nitrogen) and for other cereal products. Miss Fraser (1931) has shown that in the adult albino rat hair growth occurs continuously throughout life.

The importance of hair growth in determining the amino acid requirements for the growing rat would be much less than for the adult rat, since the most active protein growth relates to other tissues than hair in the former case. The cystine-methionine content of the proteins of these protoplasmic tissues is comparatively low, of the order of 4 to 6 gm per 16 gm of nitrogen, while the lysine content is high 7 to 8 gm on the same basis. Hair growth in the adult human would also presumably be a minor factor in determining amino acid requirements since the human body is only sparsely covered with hair. This conception of the difference between the rat and man with respect to protein nutrition was stated by Mueller and Cox (1947), and Cox *et al* (1947).

#### A THE RAIDING OF PROTOPLASMIC TISSUES FOR METHIONINE-CYSTINE

The argument just presented assumes that hair growth will continue during periods when the animal is in negative nitrogen balance, since the protein utilization of adult rats and adult humans was determined under such conditions. Long periods of protein depletion will not deplete a rat of its hair coat, probably because hair growth will continue at the expense of other tissue constituents. This transfer of amino acids from dispensable protein stores using the term in the sense that Whipple has given it may lead to an accelerated output of urinary nitrogen during specific protein nutrition in proportion to different animals to the relative intensity of hair growth. The depression in the output of endogenous urinary nitrogen induced by the ingestion of methionine has been reported in the literature for rats (Brush *et al* 1947) and dogs (Alhson *et al* 1947 and Eckert 1948), but not for man (Johnson *et al*, 1947), and may be due to the effect of this amino acid in arresting the raiding of protoplasmic tissues to secure cystine for hair growth where hair growth is a considerable item in the anabolic processes of the body.

An analogous situation was revealed by Ackerson and Blish (1925) in their study of the effect of cystine on the endogenous metabolism of molting hens. For molting hens in which feather replacement is proceeding at a rapid rate, the average daily output of nitrogen in feces and urine on a nitrogen free diet was 239 mg per kilogram of body weight. This value is considerably above the usual level of endogenous nitrogen excretion of mature hens. Another group of 6 hens of the same breed (Rhode Island Red) and size (about 2400 gm) received in addition to their nitrogen free diet 150 mg of cystine daily. The endogenous nitrogen loss in this group of birds averaged 137 mg daily per kilogram of body weight a decrease of 43% compared with the birds receiving no supplement. The decrease was highly significant statistically and seemingly represents a sparing of an accelerated protein catabolism initiated by the necessity of securing cystine from other tissues for feather growth. However the feeding of methionine to molting hens does not shorten the molting period (Taylor and Russell 1943).

### B WOOL GROWTH

The persistence of keratin synthesis in animals under conditions of undernutrition is strikingly illustrated by experiments reported from the University of Illinois on wool growth in sheep. In experiments designed to measure the relative net energy value of alfalfa, clover and timothy hay for the maintenance of sheep the consumption of hay was limited by the consumption of the least palatable one namely timothy hay. As a result 5 sheep during a feeding period of 200 days lost 20% of their initial body weight (Mitchell *et al* 1928c). On analyzing their carcasses at the termination of this period and comparing the results with the composition of check sheep analyzed at the beginning of the experiment it was found that the undernourished sheep had lost 71% of their fat, 48% of their gross energy, but only 66% of their protein although they were in continuously negative nitrogen balance. However the growth of wool as well as its proximate analysis was normal amounting to 0.134 lb of protein and 633 calories<sup>1</sup> of gross energy per day per 1000 lb live weight. A total of 961 gm of protein had been laid down in the fleece. Much of this keratin nitrogen must have been produced by raiding the protoplasmic tissues for the necessary amino acids particularly for cystine and methionine.

The relative dominance of wool growth in the protein nutrition of young growing lambs and of more mature animals is shown by two other experiments from the same laboratory (Mitchell *et al* 1926

<sup>1</sup> The word calorie as used throughout the volume designates the kilogram calorie.



1928b) carried out in much the same way except that supermaintenance rather than submaintenance rations were fed. In one experiment 9 lambs weighing 65 to 70 lb initially were fed for 131 to 141 days on a ration of alfalfa hay and corn. Their average daily gains in body weight ranged from 0.18 to 0.36 lb. On carcass analysis, wool growth was found to account for 26% of the retained nitrogen. A second group of more mature lambs, weighing initially 84 to 91 lb, were fed on alfalfa hay alone for 182 days, making average daily gains in body weight of 0.10 to 0.23 lb. Of the protein stored during this period an average of 60% was recovered in the wool growth.<sup>2</sup>

### C FURTHER EVIDENCE AND RECAPITULATION

Thus the theory that explains best the different results on protein utilization secured with growing and mature rats and with human adults is that the relative dominance of hair growth in the protein metabolism of animals may, in the main, determine the proportionate requirements of the various amino acids, because of the peculiar amino acid composition of the scleroproteins. Their richness in cystine would intensify the need for the sulfur containing amino acids to the extent that the demands of amino acids for hair growth dominate the demands for protoplasmic growth and for endogenous replacements. The richness of the keratins of feathers in arginine seems to be responsible for the accentuated demands of the chicken for this amino acid (Hegsted *et al.* 1941) the arginine requirement being greater for the more rapidly feathering breeds. The poverty of hair proteins in lysine would depress the proportionate lysine requirements. These predictions are borne out by the data presented. Keratins are low in histidine 1.0 gm per 16 gm of nitrogen and in phenylalanine 3.7 gm per 16 gm of nitrogen, in comparison with whole egg protein. If the theory is correct one would expect that the histidine and phenylalanine requirements of an animal in which the protein anabolism is dominated by hair growth (keratin synthesis) would be inconspicuous. Burroughs *et al.* (1940a,b) were able to maintain adult rats in nitrogen equilibrium for short periods by the force feeding of diets lacking in lysine, histidine and phenylalanine if tyrosine was present.

The peculiar amino acid composition of keratins in hair, nails, wool, feathers, etc., and the relative magnitude of keratin synthesis which persists throughout life may well be the most important factors in de-

<sup>2</sup> Of the same significance are the observations of Perce (1938) that fluorine concentrations in the ration of sheep that ultimately induce serious loss in body weight have no appreciable effect on wool growth.

termining differences in the proportionate amino acid requirements of animals of different species and ages

Keratin formation is also involved in the continual replacement of the epidermal cells of the skin after their removal by desquamation. The histological mechanism and some of the biochemical characteristics of the keratinization of human epidermal cells have been described by Mitoltsy and Sinesi (1957)

#### IV COMPOSITION OF TISSUE PROTEINS AND AMINO ACID REQUIREMENTS FOR GROWTH

A logical extension of the conception stated in the 1st section is that the amino acid requirements of the rapidly growing animal are largely determined in the last analysis by the amino acid composition of the tissue proteins formed during growth. In the adult animal the amino acid content of the keratin tissues constituting the integument and its appendages seems to dominate the total amino acid requirements in accordance with the relative intensity of the growth of hair wool feathers etc. as compared with the replacement of the endogenous losses of nitrogenous material in the young animal and particularly the rapidly growing animal. The amino acid analyses of groups of animal tissues assembled in Table IV should afford a test of this hypothesis

##### A SIMILARITIES IN THE AMINO ACID CONTENT OF ANIMAL TISSUE PROTEINS

The amino acid content of muscle tissue among mammalian forms of life is strikingly similar (Block and Mitchell 1946) and hence the analyses of muscles from different species have been pooled in Table IV. In fact quoting from Beich *et al* (1943) "The protein mixture which makes up voluntary muscle tissues is similar in Mammalia Aves Amphibia Pisces and Crustacea with respect to ten of the amino acids. Since muscle tissues of these various classes of animals do not differ widely in their amino acid patterns the findings support the belief that the same or closely similar amino acid composition of muscle proteins is repeated throughout the animal kingdom"

Among the visceral organs the liver kidney and brain are quite similar in amino acid composition differing from lung and stomach in higher contents of tryptophan cystine methionine and phenylalanine tyrosine. As a class the proteins of the visceral organs differ from muscle proteins chiefly in a lower content of lysine

The blood plasma proteins to which Whipple (1945) assigns such a prominent role in the protein exchanges of the body are distinguished

(Mitchell *et al*, 1928<sup>1</sup>), the nutritional value of the combined proteins may be quite low (Mitchell *et al*, 1927), due to the unique amino acid deficiencies of collagen and elastin.

### C. COMPARISON WITH GROWTH REQUIREMENTS

Since the muscles and the visceral organs contain most of the proteins in the animal body, the proportions of the essential amino acids contained in these proteins should largely determine the proportions among the requirements for these acids by the growing animal on the basis of the thesis under discussion. A test of this proposition is afforded by the data given in Table VI in which amounts of amino acids in the tissues or amounts estimated to be required are all computed using lysine as a base with a value of 100. Analogous values for whole egg proteins (Block and Mitchell, 1946) are included also, representing a dietary protein very completely utilized in metabolism.

A close correlation between tissue composition and amino acid requirements may not be revealed by the values given in Table VI because of errors in amino acid analyses but particularly because of uncertainties in estimates of amino acid requirements. Rose's values for the rat originally proclaimed in 1937 and revised slightly in 1949 (see Rose *et al*

TABLE VI

RELATIVE PROPORTIONS AMONG THE ESSENTIAL AMINO ACIDS IN ANIMAL TISSUES AND IN WHOLE EGG AS COMPARED WITH ESTIMATED REQUIREMENTS  
LYSINE IS GIVEN A VALUE OF 100

Amino acid	Muscle	Visceral organs	Whole egg	Estimated requirements	
				Rat <sup>a</sup>	Chicken <sup>b</sup>
Lysine	100	100	100	100	100
Histidine	36	47	29	40	17
Arginine	88	105	90	20	133
Phenylalanine + tyrosine	114	166	151	90	111 <sup>c</sup>
Tryptophan	16	26	21	20	20 <sup>d</sup>
Methionine + cystine	55	74	90	60	89
Threonine	57	82	68	50	50 <sup>e</sup>
Leucine	99	127	128	60	156
Isoleucine	73	87	111	50	67
Valine	74	87	101	70	84

<sup>a</sup> Rose *et al* (1949) The unconverted lysine requirement is 1.0% of the diet

<sup>b</sup> Taken from Almquist (1952) except where otherwise indicated. The unconverted lysine requirement is 0.9% of the diet

<sup>c</sup> Fisher *et al* (1957)

<sup>d</sup> Wilkening *et al* (1947)

<sup>e</sup> Grim (1949)

1949) are considered by their author as tentative their evidential support has never been revealed. The amino acid requirements of the chick may not be entirely satisfactory because of the subnormal growth generally secured by the *ad libitum* feeding of amino acid mixtures in lieu of protein and because the interpretation of the data is sometimes obscure. Nevertheless a high correlation is evident between the amino acids in muscle and the estimates of amino acid needs of the growing rat if the arginine values are disregarded because of the limited ability of the rat to synthesize this amino acid. The product moment correlation coefficient is +0.94. The correlation between the amino acid contents of visceral organs and the Rose estimates is also high with  $r = +0.83$ . The chick requirements for the essential amino acids including arginine are also highly correlated with the essential amino acids of muscle tissue ( $r = 0.85$ ). Note the high requirements of the chick for methionine, cystine and arginine as compared with the rat due in all probability to a more rapid keratin synthesis in feather growth.

These and other correlation coefficients among the variates in Table VI are assembled in Table VII. The proteins of whole egg which are

TABLE VII  
CORRELATIONS AMONG VARIATES IN TABLE VI: PRODUCT MOMENT CORRELATION COEFFICIENTS

Variates	Covariates			Amino acids required by	
	muscle	Amino acids in organs	whole egg	rats	chickens
Amino acids in muscle	—	0.67	0.92	0.94 <sup>a</sup>	0.87
organs	0.67	—	0.70	0.83 <sup>a</sup>	0.80
whole egg	0.92	0.70	—	0.81 <sup>a</sup>	0.83

<sup>a</sup> Omitting the arginine in both variates

so highly utilized in metabolism are highly correlated in their content of the essential amino acids with the proteins of muscle ( $r = 0.92$ ) for which they serve so effectively as dietary precursors. Bocobo *et al* (1952) analyzed some human tissues for amino acids and correlated the results with the amino acid contents of whole egg proteins. The correlation coefficient was +0.811. For proteins of lower biological values smaller coefficients were secured 0.791 for casein 0.516 for gluten and 0.455 for whole corn.

The evidence presented in Tables V, VI and VII proves that the requirements of the essential amino acids that must be provided preformed in the diet of the growing animal are determined to a very large extent probably to a larger extent than the cited evidence indicates by the amino acid makeup of the tissues they are producing. This fact

(Mitchell *et al* 1928a) the nutritional value of the combined proteins may be quite low (Mitchell *et al*, 1927), due to the unique amino acid deficiencies of collagen and elastin

### C COMPARISON WITH GROWTH REQUIREMENTS

Since the muscles and the visceral organs contain most of the proteins in the animal body the proportions of the essential amino acids contained in these proteins should largely determine the proportions among the requirements for these acids by the growing animal on the basis of the thesis under discussion. A test of this proposition is afforded by the data given in Table VI in which amounts of amino acids in the tissues or amounts estimated to be required are all computed using lysine as a base with a value of 100. Analogous values for whole egg proteins (Block and Mitchell, 1946) are included also representing a dietary protein very completely utilized in metabolism.

A close correlation between tissue composition and amino acid requirements may not be revealed by the values given in Table VI because of errors in amino acid analyses but particularly because of uncertainties in estimates of amino acid requirements. Rose's values for the rat originally proclaimed in 1937 and revised slightly in 1949 (see Rose *et al*,

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organs	0.67	—	0.70	0.83*	0.80
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\* Omitting the arginine in both variates

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suggests that an effective method of determining the requirements of a growing animal for these amino acids may be to determine, first, the requirement in grams per day of some one amino acid, such as lysine, and then to estimate the requirements of the others from the proportion existing between the essential amino acids and lysine in the body of the animal, these proportions to be determined by amino acid assays of the entire carcass, or approximately, even by amino acid assays of a dominant tissue such as muscle. This method may be as accurate as methods in current use for measuring amino acid requirements.<sup>3</sup> It has the distinct advantage of avoiding the confusion concerning the utilization of the isomers that are sometimes fed in racemic mixtures. The usual assumption that the utilization of these isomers is an "all or none" proposition has been shown to be wrong in some instances at least since partial utilization has been established (Rose, 1937; Durr and Bader, 1940; Albanese, 1944; Wilkening and Schweigert, 1947).

## V AMINO ACID REQUIREMENTS FOR NITROGEN EQUILIBRIUM IN THE ADULT

Estimations of the amino acid requirements of the adult organism are not in a satisfactory state even as regards the number of amino acids required. The validity of the use of nitrogen equilibrium as a criterion of amino acid adequacy has been challenged (Holt, 1944; Holt and Albanese, 1944), particularly in the case of arginine and of histidine (Albanese *et al.*, 1944). Certainly such a criterion fails to consider the adult accretion of protein material in the growth of those tissues, mainly the integumental structures, that grow throughout life. For adult man the nitrogen equivalent of adult growth has been assessed at about 0.56 gm per square meter of body surface daily (Mitchell, 1949). For adult women two values may be computed from nitrogen metabolism data: one value of 0.37 gm of nitrogen per square meter body surface per day is based upon the report of Bricker and others (1949) of metabolism tests on 10 college women for a period of 10 weeks at constant body weight, and the other 0.42 gm per square meter per day is based upon metabolism tests on 8 young women for 12 weeks described by Johnston and

<sup>3</sup> The method has been applied by H. H. Williams *et al.* (*J. Biol. Chem.* 208: 277-286, 1954) to the determination of the amino acid requirements for growth of the rat, the chick, and the pig. Carcass analyses at different ages revealed a remarkably similar pattern of amino acid composition among the three species and within each species at different ages. Also the requirements determined by nutrition studies were similar among the species and in close agreement with the requirements calculated by carcass analysis. The authors conclude that the carcass analysis procedure is a valid method for evaluating the growth requirements for most if not all of the essential amino acids.

McMillan (1952) The three results, 0.56, 0.37 and 0.42 gm of nitrogen per square meter of body surface per day suggest that the male adult has a somewhat more rapid rate of adult growth than does the female.

In evaluating nitrogen balance studies the dermal loss of nitrogen must also be considered although it rarely is. Insensible perspiration contains appreciable quantities of nitrogen, 180 to 230 mg per day, according to Freyberg and Grant (1937). Under minimal sweating conditions this dermal loss may amount to 0.36 gm of nitrogen daily (Mitchell *et al.* 1949). When sweating occurs, whether psychic or thermic in origin, the loss of nitrogen (Mitchell *et al.*, 1949; Cuthbertson and Guthrie 1934) and of amino acids (Hier *et al.* 1946) will be greatly increased.

Thus amino acid requirements in adult animals assessed by nitrogen balance studies are minimal figures but they may serve the purpose of comparing the magnitudes of the requirements of the different essential amino acids using this term to cover the 10 amino acids originally shown by Rose to be essential for normal growth. The experiments of Burroughs *et al.* (1940) indicate that the requirements of the adult rat for lysine, histidine and phenylalanine are inconspicuous in magnitude, probably because of the dominance of keratin synthesis in the protein nutrition of the adult rat.

The values given in Table III on the absorbed nitrogen per basal calorie from different proteins and protein foods required for nitrogen equilibrium in the adult rat and in adult man together with the determined contents of these protein foods in the essential amino acids per 16 gm of nitrogen (Block and Mitchell 1946) have been used to compute the daily requirements of the individual amino acids for a 325 gm rat and 70 kg man. For each protein for which a requirement is given in Table III the corresponding amounts of the different essential amino acids were listed both for the rat and for man. The required amount for each amino acid in each case is then taken as the smallest amount in the list. The requirements thus obtained are summarized in Table VIII.<sup>4</sup>

The requirements for adult man are listed side by side with those recently proclaimed by Rose (1957). The two may not be comparable first because of the indirect method of estimation used to secure the values given in column 3 of the table and second because Rose's values although described as "minimum daily requirements" are not averages of the 3 to 6 individual determinations for the different amino acids and hence are not the most probable requirements for any given indi-

<sup>4</sup> Essentially the same method was used by Harte R. A. and Travers J. J. (Science 105: 15, 1947) in estimating the amino acid requirements for adult man from less extensive data.



vidual or group of individuals. They are the highest of the group of determinations in each case. It would be expected that the range between the most probable requirement (the average) and the highest requirement (the one selected) would be the greater the larger the group of subjects tested.

TABLE VIII  
ESTIMATED DAILY AMINO ACID REQUIREMENTS FOR THE ADULT RAT<sup>a</sup> AND THE ADULT MAN<sup>b</sup>

Amino acids	Estimated requirements for the rat <sup>c</sup> (mg)	Requirements for adult man	
		Estimated <sup>d</sup> (gm)	Reported by Rose (1957) (gm)
Arginine	21.9	1.18	0
Histidine	7.8	0.55	0
Lysine	11.3	0.99	0.80
Phenylalanine tyrosine	40.3	2.42	1.10
Tryptophan	5.6 <sup>d</sup>	0.32	0.25
Methionine cystine	19.1	1.05	1.10
Threonine	12.2	0.56	0.50
Leucine	34.3	1.86	1.10
Isoleucine	20.8	1.12	0.70
Valine	23.6	1.18	0.80

<sup>a</sup> For male albino rat weighing 325 gm with a basal metabolism of 27.6 cal per day

<sup>b</sup> For a man 70 kg in weight 174 cm in height 25 years of age with a basal metabolism of 1700 cal per day

<sup>c</sup> Based upon data in Table III and the amino acid composition of the respective proteins (Block and Mitchell 1946) as explained in the text

<sup>d</sup> Estimated from nitrogen balance studies at 4.0 to 6.4 mg for a 200 gm rat by A. A. Wykes, L. M. Henderson and C. A. Elvehjem (*J. Nutrition* 40: 71, 1950)

Rose (1957) has compared his "minimum" amino acid requirements for men with those obtained with young women by Leverton and associates at the University of Nebraska and by Swendseid and Dunn at the University of California in Los Angeles. The values given for women are generally, when comparable, less than the corresponding values for men. This is particularly true for the leucine requirements. It seems doubtful that Rose's criticisms of valid of the experimental procedures used in the laboratories other than his own would account for the sex differences observed for the various amino acids. Nor are the differences in body weights between the men and women subjects great enough apparently to explain the discrepancies, especially since none of the investigators in this field except Clark *et al.* (1957) at Purdue University has been able to detect a correlation between amino acid

requirement and body size among their samples of the human population. The writer hesitates, however, to conclude that there are considerable differences between the sexes in their requirements for the essential amino acids in adult nutrition. The answer to this problem may be elicited only when adequate groups of men and women are compared with the same diets and under the same experimental conditions. The development of a logical method of expressing amino acid requirements with reference to body size and caloric needs and particularly the comparison of *average* requirements with due regard to their standard errors, rather than extreme values whose random errors cannot be estimated, would represent a marked advance in interpretive procedures.

The estimated values given in Table VIII would be expected from the method of their calculation to be nearer the minimum values for those amino acids in which the basic proteins are deficient, i.e. for lysine and for methionine cystine. The values for the other amino acids may be larger than the true requirement either because they are contained in all of the basic proteins in amounts larger than those required or because the amounts derived by the method employed include certain fractions used for the synthesis of the nonessential amino acids. It may be more of a coincidence than otherwise that the estimated human requirements for lysine and methionine cystine as well as those for tryptophan and threonine are of the same order of magnitude as the "minimum requirements" announced by Rose.

Finally, it may be noted from the estimated requirements given in columns 2 and 3 that the ratio of the methionine cystine to the lysine requirement is much greater for the adult rat than for the adult human, i.e. 1.71 and 1.06 respectively. This relationship conforms with expectation in view of the probable greater dominance of keratin synthesis in the protein nutrition of the adult rat than in that of the adult human.

## VI A THEORY OF PROTEIN METABOLISM

An important step in the development of a science is the incorporation of new ideas and facts into its body of laws and principles. Of no less importance is the harmonizing of conflicting theories and interpretations of facts. The facts, if they are such, cannot be in conflict.

The present status of the theories of protein metabolism is not a harmonious one. A rediscussion of the problem is therefore in order.

### A BASIC PRINCIPLES

The experimental investigations of Folin on the effect of the protein level of the diet on the composition of human urine (1905a) mark the

beginning of modern concepts of protein nutrition. The facts thus established are still valid. Their interpretation by Folin (1905b) was a logical one and to a large degree the essential points of this interpretation are valid today and can successfully withstand criticism. The basis of Folin's theory of protein metabolism can best be presented by a few quotations from his classical paper (1905b).

We have seen from the tables that the composition of urine, representing 15 gm of nitrogen or about 95 gm of protein differs very widely from the composition of urine representing only 3 gm or 4 gm of nitrogen, and that there is a gradual and regular transition from the one to the other. To explain such changes in the composition of the urine on the basis of protein katabolism, we are forced it seems to me, to assume that katabolism is not all of one kind. There must be at least two kinds. Moreover from the nature of the changes in the distribution of the urinary constituents it can be affirmed I think, that the two forms of protein katabolism are essentially independent and quite different. One kind is extremely variable in quantity, the other tends to remain constant. The one kind yields chiefly urea and inorganic sulphates, no kreatinin and probably no neutral sulphur. The other, the constant katabolism is largely represented by kreatinin and neutral sulphur and to a less extent by uric acid and ethereal sulphates. The more the total katabolism is reduced the more prominent become these representatives of the constant katabolism, the less prominent become the two chief representatives of the variable katabolism.

The fact that the kreatinin elimination is not diminished when practically no protein is furnished with the food and that the elimination of some of the other constituents is only a little reduced under such conditions shows why a certain amount of protein must be furnished with the food if nitrogen equilibrium is to be maintained. It is clear that the metabolic processes resulting in the end products which tend to be constant in quantity appear to be indispensable for the continuation of life, or to be more definite those metabolic processes probably constitute an essential part of the activity which distinguishes living cells from dead ones. I would therefore call the protein metabolism which tends to be constant, *tissue metabolism* or *endogenous metabolism* and the other the variable protein metabolism I would call the *exogenous* or *intermediate metabolism*.

"The exogenous or intermediate protein katabolism is here conceived as consisting of a series of hydrolytic splittings resulting in a rapid elimination of the protein nitrogen as urea.

Later advances in our knowledge of protein metabolism relate mainly to the details of the processes of protein assimilation and protein utilization.

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## B THE ENDOGENOUS PROTEIN CATABOLISM

The essential constancy of the endogenous protein catabolism has been repeatedly confirmed and its approximately constant relationship to the basal energy metabolism has been established (Mitchell and Beldes 1950 and literature there cited Palmer *et al*, 1914 Mukherjee and Mitchell, 1949 Blaxter and Wood 1951) as well as the conditions disturbing this relationship (Treichler 1939 Treichler and Mitchell, 1941) The independence of the endogenous and the exogenous metabolism of nitrogen was confirmed experimentally by Burroughs *et al* (1940)

Of particular significance is the work of Schoenheimer's group on the metabolism of creatine using the isotope technique They showed (Bloch *et al* 1941) that muscle creatine is dehydrated to creatinine at a very constant rate and "is not involved in any biological reaction in which linkages between carbon and carbon and carbon and nitrogen are broken" "It thus differs in its metabolic aspects from all other biological compounds so far investigated with isotopes"

Later isotope studies of the same problem cited by Mitchell (1955) have produced evidence of a dichotomy in the total metabolism of organic nutrients in the body similar to Folin's proposal of a dichotomy in nitrogen metabolism into an endogenous and an exogenous fraction The article (Mitchell 1955) also presents further evidence in favor of the essential features of Folin's theory The publication of Still (1957) on the amino acid turnover in the brain of the mouse compared with that of other tissues has been interpreted by the author as being "consonant with Folin's concept of endogenous and exogenous metabolism"

## C PROTEIN STORES IN THE BODY

Whipple and his group have established the existence of dispensable and indispensable stores of protein in the animal body (Whipple 1948) in the course of their work on hemoglobin and blood plasma protein regeneration Dispensable stores are readily raided when emergency demands for specific amino acids or for protein are not covered by the food supply The forms in which these protein stores exist is not known Their ready mobility apparently depends upon their exposed position in the cells rather than upon their chemical nature an analogy would be the ready accessibility of the calcium salts in the epiphysis as contrasted with the diaphysis of the long bones (Bauer *et al* 1929) While the location of the protein stores is probably quite general the outstand

beginning of modern concepts of protein nutrition. The facts thus established are still valid. Their interpretation by Folin (1905b) was a logical one and to a large degree the essential points of this interpretation are valid today and can successfully withstand criticism. The basis of Folin's theory of protein metabolism can best be presented by a few quotations from his classical paper (1905b).

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ing role of the liver in the capacity of a storage organ seems clear (Addis *et al* 1940, Kosterlitz and Campbell 1945 Kosterlitz 1947)

The dispensable protein stores rise and fall as the protein intake rises or falls. While these stores appear to contribute nothing to the normal functioning of the body cells they do exert a protective function when the body is subjected to the strain of chloroform or arsphenamine poisoning and other destructive agencies, and in Whipple's words they are a bulwark against infection. They bear the brunt of sudden increases in protein catabolism due to inflammation, hyperthyroidism and probably other stresses.

#### D THE ROLE OF THE BLOOD PLASMA PROTEINS

Before it was shown experimentally that the amino acids resulting from protein digestion gained access to the blood as such, Abderhalden and London (1910) proposed the theory that, in their passage through the intestinal mucosa, the proteinogenous amino acids were combined to form the blood plasma proteins which in turn nourished the cells of the body. The theory served a good purpose in explaining the facts available at the time it was elaborated. It was rightly discarded when the occurrence of hyperaminoacidemia during protein digestion was established.

It is a matter of considerable historical interest that the theory in some of its features has been revived mainly as a result of the work of Whipple (1948). The ability of the blood plasma proteins to nourish the other cells of the body is evident from the fact that an animal may be kept in nitrogen equilibrium by the intravenous injection of compatible blood plasma as the sole source of nitrogen (Elman 1944). The conversion of blood plasma protein into cytoplasmic protein occurs apparently without prior degradation into amino acids, since phlorizinized dogs thus nourished do not excrete extra sugar in the urine. According to Howland and Hawkins (1938)

The metabolism of protein when fed is different than when it is injected. It is suggested that there is a partial catabolism of the injected protein with reassembly of the large aggregates formed by the cells to form their own peculiar type of protein.

This partial catabolism with reassembly of the large aggregates may be the method of protein interchange within the body rather than a complete catabolism to amino acids with subsequent resynthesis to protein.

Reineke *et al* (1941) provide additional evidence of this type of protein interchange in their demonstration that milk globulin is formed

in the mammary gland of the goat from a globulin glycoprotein of the blood plasma

Ebert (1954) has reviewed later evidence "for the transfer of protein from plasma to fixed tissues followed by transformation of the plasma protein to other kinds of intracellular protein

### E KERATIN SYNTHESIS

The peculiar amino acid demands for keratin synthesis due to the unique amino acid composition of keratins together with the persistence of this synthesis under conditions of protein under nutrition at the expense of protoplasmic proteins in other tissues has been fully established and discussed above. Its dominance in the protein nutrition of maturity in those animals supporting a full coat of hair, wool fur, or feathers is to be expected

### F THE ENDOGENOUS PROTEIN CATABOLISM STUDIED BY THE ISOTOPE TECHNIQUE

The investigations of Schoenheimer and his group at Columbia University on intermediary protein metabolism using amino acids labeled with  $N^{15}$  have revolutionized conceptions of the exogenous metabolism of Folin. They have revealed a dynamic rather than a static state of the tissue proteins (Schoenheimer 1942) and have shown "that all reactions for which specific enzymes and substrates exist in the animal are carried out continuously. Quoting further from the writings of this group (Schoenheimer *et al.* 1939)

"It has been shown that nitrogenous groupings of tissue proteins are constantly involved in chemical reactions: peptide linkages open the amino acids liberated mix with others of the same species of whatever source: diet or tissue. This mixture of amino acid molecules while in the free state takes part in a variety of chemical reactions: some reenter directly into vacant positions left open by the rupture of peptide linkages; others transfer their nitrogen to deaminated molecules to form new amino acids. These in turn continuously enter the same chemical cycles which render the source of the nitrogen indistinguishable. Some body constituents like glutamic and aspartic acids and some proteins like those of liver serum and other organs are more actively involved than others in this general metabolic mixing process. The excreted nitrogen may be considered as a part of the metabolic pool originating from interaction of dietary nitrogen with the relatively large quantities of reactive tissue nitrogen.

Thus our knowledge of the nature of intermediary protein metabolism has undergone a profound change since the time of Folin. But



as far as end results are concerned these isotope studies have not changed the Folin conception of the exogenous metabolism in the slightest. These reversible reactions revealed by isotope studies between tissue proteins and dietary amino acids are not anarchistic in nature. They seem to represent automatic and non interruptable biochemical processes, of synthesis as well as degradation which are balanced by an unknown regulatory mechanism so that the total amount of the body material and its composition do not change. (Moss and Schoenheimer 1940). Hence for the purposes of this discussion the tissue proteins may be considered to be static.

Furthermore the magnitude of these intermediary reactions and in particular the amount of nitrogen excreted in the urine as a result is determined primarily by the magnitude of the nitrogen intake of the animal. Urea and ammonia are still the primary end products that appear in the urine. These reversible reactions, which may according to Sprinson and Rittenberg (1949) involve only a fraction of the muscle proteins possibly only dispensable protein in the Whipple sense of the term, are sharply distinguished from the irreversible reactions involving tissue proteins and other nitrogenous constituents, characteristic of the endogenous catabolism of Folin and typified by the creatine dehydration to creatinine.

The main effect of these isotope studies of the intermediary protein metabolism is to render the term exogenous somewhat, though not completely inappropriate. It is still the body's method of ridding itself of nitrogen consumed in amounts exceeding its needs for endogenous replacement and for growth, nitrogen being the one element in the protein molecule that it cannot oxidize.

## VII A SCHEMATIC REPRESENTATION OF PROTEIN METABOLISM

Figure 1 is an attempt to combine into one picture the facts and the modern theories of protein metabolism and protein assimilation. The diagram consists of a series of cells bearing labels possessing a biochemical or functional rather than an anatomical significance grouped about the metabolic pool of amino acids which has been defined by Sprinson and Rittenberg (1949) in the following terms:

We shall here define the metabolic pool of the animal (or organ or cell) as that mixture of compounds derived either from the diet or from the breakdown of the tissues which the animal (or organ or cell) employs for the synthesis of tissue constituents. The nitrogenous compounds of the metabolic pool constitute the nitrogen pool.

It is probable that all of the constituents of the nitrogen pool are present in the non protein fraction. The reverse is not true. Urea and

creatinine for example are not part of the metabolic pool since they are solely excretory products

The flow lines identified by numbers in circles originate with the "dietary protein" cell at the top of the diagram and may be described briefly as follows

- (1) Incomplete digestion of dietary protein
- (2) Gastrointestinal proteolysis absorption into the blood and transportation to the tissues where the resulting amino acids are incorporated into the metabolic pool

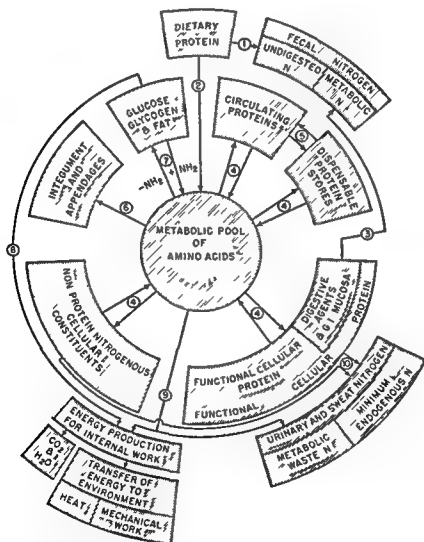


FIG 1 A diagrammatic representation of protein metabolism in a sexually inactive animal. The significance of the numbers on the flow lines are explained in the text

(3) Loss of body nitrogen in the feces, the so called metabolic fecal nitrogen. This nitrogen originates within the body of the animal and is contained in the unabsorbed constituents of the digestive juices and cellular material (Leblond and Stevens 1948), and mucus derived from the gastric and intestinal mucosa. Its significance and importance in ruminant nutrition have been discussed by Blaxter and Mitchell (1948). Its replacement is essential to the maintenance of the nitrogenous integrity of the body and it is hence an item in the protein requirement of the animal. That this replacement occurs under normal nutritional conditions is clearly shown by the observations of Tarver and Schmidt (1952) and of Friedberg (1947) using dietary amino acids labeled with radioactive isotopes of sulfur or carbon. These studies revealed that protein synthesis was most active in the intestinal wall and in the pancreas.

The intestinal wall secretes enzymes and mucous proteins which are lost in enormous quantities (unlike other enzymes and proteins within the body). To compensate for this loss the intestinal wall may be more active in protein synthesis than other organs. If this explanation is correct, then the pancreas should also show a high turn over rate. Pancreatic juice contributes many enzymes needed for digestion. Actually Tarver and Schmidt found that the pancreas has the second highest specific activity among the organs of animals treated with isotopic methionine (Friedberg 1947).

(4) The reversible reactions occurring continuously on a protein containing diet between the dietary amino acids, on the one hand, and the body proteins and nonprotein nitrogenous constituents, on the other hand. These are the reactions revealed by the isotope technique of Schoenheimer and his associates.

(5) These reactions between the circulating proteins of the blood, mainly the blood plasma proteins, and the dispensable protein stores in all tissues of the body have been described by Whipple (1948).

(6) The synthesis of the keratins of hair, wool, epidermis, nails, claws, hoofs, etc. The continued growth and regeneration of these protective proteins against environmental stress are sufficiently important to the body that, in case the food supply is inadequate to support them, the protoplasmic tissues of the body are raided to supply the amino acids, particularly methionine-cystine needed via the metabolic pool. In the adult animal the protein requirements relate to the replacement of metabolic nitrogen lost in the feces (Block and Mitchell, 1946), the endogenous losses in the urine (Smuts 1935), and the growth of the integument and its appendages (Mitchell, 1949). If the animal is of the fur/hairness type according to the Friedenthal classification (Fraser

1931) like the albino rat the amino acid requirements for keratin synthesis will dominate the total amino acid requirements

(7) The storage of protein energy, after denitrogenation as glycogen and fat. These reactions are reversible if only to a limited extent the amino group being provided by the metabolic pool

(8) The oxidation of proteinogenous glycogen and fat to provide energy for internal work later dissipated as heat and for mechanical work

(9) The direct oxidation of amino acids for energy production also some direct leakage of amino acids through kidney sweat glands and possibly epidermis in the insensible perspiration

(10) The irreversible reactions involving functional cellular proteins the indispensable proteins of Whipple (1948) and the nonprotein nitrogenous constituents of the cells preeminently creatine constituting the endogenous catabolism of Folin. The replacement of these losses and of the metabolic fecal losses together with keratin synthesis (Lardy and Feldott 1949) and other types of adult growth constitute the maintenance protein requirement of the adult animal in a nonstress environment. When the energy supply of the animal is inadequate and the deficiency cannot be currently supplied by body glycogen or fat or under stress conditions involving hyperthyroid activity (Mukherjee and Mitchell 1949) and possibly other endocrine disturbances the endogenous catabolism is accelerated and generally a cretinuria results

### VIII SUMMARY

From a study of the utilization by the growing rat the adult rat and adult man of the nitrogen of a series of 6 proteins possessing limiting deficiencies of either lysine or methionine cystine it was shown that the biological values were higher for the adult rat than for the growing rat and for adult man when the dietary protein was deficient in lysine and they were lower when the dietary protein was deficient in methionine cystine

The proteins of whole egg prepared in the laboratory appear to be slightly deficient in lysine for maximum utilization by the growing rat

When the utilization of the nitrogen of the 6 proteins tested in adult nutrition are expressed as the amount of absorbed nitrogen per calorie of basal heat required for nitrogen equilibrium the values are of the same order of magnitude for the rat and the human but are definitely larger for the rat in the case of proteins deficient for growth in methionine cystine and definitely smaller in the case of proteins deficient for growth in lysine

These relationships indicate that the cystine methionine requirement

is relatively more intense for the adult rat than for either the growing rat or for the adult human. On the other hand, the lysine requirement seems to be much less prominent among the amino acid requirements of the adult rat than among those of the growing rat or the adult human.

The reason for these relationships appears to be traceable to the relative prominence of keratin synthesis for the growth of the integument and its appendages (hair, wool, etc.) In the adult rat, in which protoplasmic growth is minimal but hair growth over the entire body continues, keratin synthesis with its high requirement for cystine and its low requirement for lysine, histidine, and phenylalanine, dominates the total amino acid requirements.

This conception of the importance of keratin synthesis in determining the amino acid requirements of animals is fortified by evidence obtained from studies of feather growth in molting hens and wool growth in sheep.

A corollary from this conception is that the total amino acid requirements of an animal of any age or species are determined by the proportions of the essential amino acids contained in the tissues being currently formed, or being currently catabolized.

In partial confirmation of this theory, striking similarities and high correlations are shown to exist between the proportions existing among the essential amino acids of the dominant tissues of the animal body and the proportionate requirements for these amino acids as determined by feeding experiments with rats and chicks.

A method is proposed and illustrated for approximating the amino acid requirements for maintenance from the amounts of absorbed nitrogen per basal calorie required for nitrogen equilibrium in the form of a series of proteins of variable value in adult nutrition.

Finally, a theory of protein metabolism is proposed, and illustrated by diagram. This theory is based upon the Folin conception of two distinct types of protein catabolism but includes later developments especially those of Whipple and Schoenheimer and their associates and the developments presented in this chapter particularly the role of keratin synthesis.

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## CHAPTER 3

# Individuality of Amino Acid Needs

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## I INTRODUCTION

The topic of our discussion is intimately related to the much broader subject of biochemical individuality which has recently been treated in a separate volume (Williams 1956a). The extremely diverse evidence with respect to the scope and magnitude of the biochemical differences which are to be found in normal human bodies must be considered as a whole before the significance and importance of any part of the evidence can be properly adjudicated. Thus our relatively meager knowledge about individuality of amino acid needs gains much in significance when we know that this is only one area among dozens in which large differences exist.

The importance of the phenomenon of biochemical individuality rests like the idea of organic evolution upon many corroborative evidences which separately might be disregarded by conservative scholars who are not anxious to incorporate new complexities into their thinking. Cumulatively however these evidences are overwhelming and the material which we will present on individuality in amino acid needs gains strength when considered in the light of a large body of other related evidence.

## II GENETIC BASIS FOR INDIVIDUALITY IN NEEDS

The fundamental clear demonstration of gene enzyme relationships made by Beadle (1945) has been productive of a large volume of research all of which emphasizes the unity of the biological world and

the extension of genetic concepts to all forms of life. From this extensive investigation we can safely draw the broad conclusion expressed by Wagner and Mitchell (1955a) that genes exert their effects through control of metabolism. This leads us to conclude that no two individuals can have identical metabolisms unless they have identical inheritance and that each member of the human family has a distinctive metabolism and probably a distinctive amino acid metabolism. If this probability is a reality, we must conclude that each individual has amino acid needs which are quantitatively distinctive. This line of argument has nothing to do with the magnitude of the differences, they may be large or insignificantly small so far as this part of our discussion is concerned.

A most important consideration which bears on our problem is the common occurrence in all forms of life of "partial genetic blocks" (Mitchell and Houlahan 1946) or their equivalent. As a result of their discovery there has been a substantial modification of our ideas with respect to the nature and character of mutations. It was at first supposed that irradiation, for example, simply cancelled out certain genes and that the observed metabolic effects were the result of complete destruction, from the functional viewpoint of these individual genes. It became evident however from investigations following the original classic ones that genes may be modified to many different degrees, and that partial impairment is a far more common phenomenon than complete destruction. The first observation with respect to partial blocks or "leaky genes" was that while a mutant strain of *Neurospora* was quite unable to grow at 35°C unless riboflavin was added to its culture medium, the potentiality for synthesizing riboflavin for its own needs was not actually lost. At lower temperatures the potentiality was shown to exist; it had only been impaired or modified. Wagner and Mitchell (1955b) state: "It is highly probable that every gene has the potentiality of mutating to a very large number of different states." The possibilities may be graded into a spectrum.

This important concept leads one to suppose that the metabolism of a particular amino acid, for example (including both anabolism and catabolism) where a number of different pathways and a larger number of steps are involved, could take place in two individuals in accordance with strikingly different overall patterns, not because of the lack of specific genes in the make up of either individual but because of quantitative differences occasioned by the numerous different states in which each of the specific genes can exist.

Genetic considerations such as these which we cannot discuss in detail would lead us to presuppose even before any direct evidence was

at hand that human individuals would have quantitatively distinctive amino acid needs. How great the distinctive differences might be is another question—one which we could not answer *a priori*.

### III ANATOMICAL AND COMPOSITIONAL BASIS FOR INDIVIDUAL NEEDS

If one typical adult human body has substantially the same make up as another—with organs and other structures all substantially the same both as to morphology and chemical composition—then we might suppose that the amino acid needs would be substantially the same for each body. Amino acids are required fundamentally to maintain the proteins in our bodies and if these proteins are qualitatively and quantitatively about the same it might be supposed that the needs of one body would about duplicate the needs of another.

There is ample reason however for supposing that the amino acids needed for the maintenance of one body might be appreciably different from that of another on anatomical and compositional grounds alone. According to a study of the anatomies of 645 normal male rabbits of the same stock Wade Brown and co workers (1926) found that in the group the organ weights including endocrine glands corrected for differences in total body weight varied from 2.3 fold up to 80 fold! The average variation for the 17 items measured was 14.4 fold the median variation was 10 fold and there were only two organs heart and brain for which the variation was less than 5 fold.

There is ample reason to suppose that the variations in human bodies are of the same order as that observed in rabbits. The concept that a typical body would be approximately average with respect to each organ size is completely untenable (Williams 1957). On the basis of the different protein make up of each organ coupled with distinctive patterns of organ sizes one would be led to assume that the amino acid needs of individuals would show quantitative variation.

Not only are there wide differences in organ size but there are likewise wide variations in microscopic anatomy (of the blood and bone marrow for example) which would lead supposedly to some differences in amino acid needs. The fact that each individual has distinctive blood and tissue proteins as demonstrated by transfusions and transplantation as well as electrophoretic studies is one which might occasion differences in amino acid needs but it is not clear that these would be large enough to be significant.

No data are available as to quantitative differences in the total amino acid make up of human bodies but there is evidence which we cannot detail here that specific proteins particularly the enzymes and those

hormones which are protein in nature vary in level through ranges of several fold (Williams 1956b)

Some enzymes which have to do specifically with amino acid metabolism are also known to vary from one normal individual to another. Arginase activity in erythrocytes varies over at least a 4 fold range from one well person to another (Clark and Beck 1949) and arginase in the skin varies similarly (Van Scott 1951). This strongly suggests that arginine for example may not enter into metabolism with anything like equal facility in different individuals. Several peptidases in erythrocytes were likewise found to vary among 10 individuals over ranges from 2 fold to 6 fold (Adams *et al.*, 1952). For many enzymes we have no information on the question of inter individual variation.

A point of view which needs to be brought into this discussion is that amino acid (or other) nutrition is not for the body as a whole but rather for every cell, tissue and organ within our bodies. Specialized cells and tissues have specialized nutritional requirements and we should expect nutritional needs to differ from individual to individual because of differences in the sizes and cellular compositions of the various organs and tissues. It is not by any means preposterous to suppose that some individuals may because of unusually ineffective machinery for producing a particular protein hormone have relatively high nutritional needs for crucial amino acids which enter into the composition of that hormone. The specific amino acid requirement might be completely out of line with the actual amount entering the hormone because the entire metabolic pool in the body might have to be maintained at a relatively high level in order that the limiting organ or group of cells be furnished with a sufficiently high concentration.

It is entirely possible furthermore, that real competition for specific amino acids may exist between various organs and tissues. It may be that an individual possesses an unusually high requirement for a specific amino acid by reason of the existence of a highly effective mechanism for metabolizing this amino acid in a location well removed from the point where its presence may be a limiting factor for cell activity.

#### IV DISTINCTIVE AMINO ACID PATTERNS

What may be regarded as indirect evidence with respect to variability of amino acid needs is the existence of characteristic concentration patterns of these substances in body fluids.

##### A URINARY PATTERNS

By the use of paper chromatography ascending technique (Williams and Kirby 1948) it has been found in our laboratories that each indi-

individual exhibits a highly characteristic urinary excretion pattern with respect to the amino acids which are present in urine in small amounts. In one of a number of studies (Berry *et al* 1951), from 13 to 54 samples of urine were collected from each of 6 individuals and analyzed for alanine, glycine, serine, glutamic acid and lysine. The average values for the different individuals showed a spread of 3 fold, 7 fold, 5 fold, 3 fold and over 100 fold respectively for the 5 amino acids listed. Identical twins included in the group of 6 excreted about the same amounts of each. The maximum ratio between the excretion of the twins was 1.6 and the average variation was about 30%.

In view of the fact that uniformizing the diets did not obliterate the patterns in humans (Thompson and Kirby 1949, Sutton, 1951) or rats (Reed 1951) and that they are observed in small babies (Berry and Cain 1951) there are various possible interpretations of their existence: one is that they are merely reflections of kidney differences—of the possibility that the renal thresholds for individual amino acids vary from individual to individual. The losses to the body incidentally are small and there is no substantial wastage. That kidney differences are not alone involved is indicated by the existence of similar patterns elsewhere in body fluids where kidney thresholds would not be operative. Also pointing in the same direction is the fact that the young woman (1 of the 6 above) who was found to excrete about 19 times as much urinary lysine as another of the same sex was in a later study found to secrete 10 times as much lysine in her saliva as did the other young woman (Berry 1951).

The other obvious interpretation of these urinary patterns is that the differences are a reflection possibly only a dim and distorted one of genetic differences in the completeness with which various amino acids are used up in the day to day enzyme controlled total metabolism of the individual.

## B SALIVARY PATTERNS

Distinctive patterns with respect to amino acid content are also to be found in the salivas of different individuals (Berry 1951). When samples of morning saliva (no stimulus to induce its flow) were collected from 9 different individuals and analyzed for 6 amino acids the following results were obtained (Table I). In 2 cases over 20 samples of saliva were collected from each individual and the distinctiveness of each individual was clearly evident. The most striking difference was that of lysine for which the average secretion (21 and 24 samples respectively) was 15.0  $\mu\text{g}$  per milliliter in one case and 1.5  $\mu\text{g}$  per milliliter in the other.

## C DUODENAL JUICE PATTERNS

Russel and Wewalka (1952a) applied paper chromatography to the study of the free amino acids in the duodenal juice from 10 well individuals and (1952b) 56 patients with various pathologies including those involving gall bladder and liver, and found each individual exhibited a distinctive pattern. Among the 10 normal individuals certain amino acids viz, leucine plus isoleucine, valine, alanine, glycine, and serine were generally present but the concentrations varied over a 2.5-fold

TABLE I  
SALIVARY AMINO ACIDS (9 INDIVIDUALS)

Amino acid	Range of secretion ( $\mu\text{g/ml}$ )	Number of individuals secreting detectable amounts
Aspartic acid	0-33	8
Glutamic acid	0-20	8
Serine	0-12	4
Glycine	0-36	8
Alanine	0-29	8
Lysine	0-15	4

range. Tyrosine, glutamic acid, aspartic acid, lysine, and threonine were almost always present but varied over about a 6-fold range. Glutamine, citrulline, cystine, and arginine, were found in varying amounts in about one half of the cases. Hydroxyproline, phenylalanine, and histidine were seldom present.

## D AMINO ACIDS IN BLOOD

For obvious reasons the determination of small amounts of individual amino acids in blood is difficult and we know of no study in which are recorded repeated determinations on a series of individuals. In Table II are given the ranges in the levels found in the plasmas of 17 fasting males. From these data and taking into account the information regarding other body fluids it appears probable that each individual tends to maintain a distinctive pattern of amino acids in his blood. This is made more probable by the fact that distinctive patterns of other blood components such as glucose, lactic acid, creatinine, urea, uric acid, sodium, potassium, calcium, and magnesium have been observed when repeated samples from the same individuals were analyzed (Williams *et al.* 1955). The earlier finding of Rosahn and Casey (1936) that each of 5 individuals exhibited over a period of a year a distinctive blood morphology is also in line with this probability.

## V QUANTITATIVE DATA WITH RESPECT TO INDIVIDUALITY IN NEEDS

Since an individual's needs will doubtless vary depending on various factors including especially the components of the diet, the only data which can be considered crucial with respect to individual differences in amino acid needs are those collected under comparable conditions, generally in the same laboratory.

TABLE II  
RANGES IN AMINO ACIDS IN BLOOD PLASMA<sup>a</sup>

Amino acid	Range (mg/100 ml)
Alanine	24 - 76
Lysine	23 - 58
Valine	25 - 42
Cysteine-cystine	18 - 50
Glycine	08 - 54
Proline	15 - 57
Leucine	10 - 52
Isoleucine	12 - 42
Arginine	12 - 30
Histidine	10 - 38
Threonine	09 - 36
Phenylalanine	11 - 40
Tryptophan	09 - 30
Serine	03 - 20
Tyrosine	09 - 24
Methionine	025- 10
Glutamine	40 - 106
Glutamic acid	00 - 13
Aspartic acid	00 - 12

<sup>a</sup> From Harold A. Harper, Maxine E. Hutchin and Joe R. Kummel *Proc Soc Exptl Biol Med* 80:770 (1952)

The first conclusive evidence as to individuality of amino acid needs was found by Rose (1949a). Initially it was supposed that by studying quantitatively the needs of 2 individuals the needs of "man" could be determined. This was found not to be true. In the case of some of the essential amino acids it was found by nitrogen equilibrium studies that results were consistent from one subject to another. In other cases, however, "the quantities found necessary vary as much as 100% in different individuals." In the initial experiments with tryptophan the first 2 subjects yielded approximately identical results; the minimum requirement was tentatively set at 0.15 gm per day. Later, however, a subject was encountered who required 0.25 gm per day to maintain equilibrium.



We are not here discussing the question of methodology or whether there is other evidence with respect to amino acid needs which is in conflict with that cited in this chapter. We are concerned only with individuality in needs and think it is only safe to assume, until evidence is available to the contrary, that every valid method for ascertaining individual needs at different ages, will when applied, reveal about the same degree of individuality as that arrived at by the studies cited.

An important conclusion from the author's point of view is that it is unsafe to assume that any individual has about average amino acid needs. The chance that any randomly selected individual will be in the median third of the population with respect to the needs for one amino acid is one in three. The chance that he will be in the median third with respect to 8 amino acids is about 1 in 6561. If the needs for the respective amino acids are not wholly independent, then this chance would be increased somewhat but not enough to alter one's basic thinking on the subject.

## VI DO INDIVIDUAL NEEDS DIFFER QUALITATIVELY?

There are many indirect evidences which indicate that some specific amino acids which have not been found to be essential nutritionally for adults, may nevertheless be needed by some individuals for maximum health. Since no direct attack on this problem has been made, it seems unwise to speculate at length.

Arginine has been borderline with respect to its nutritional essentiality for experimental animals. It is not commonly needed by adults for maintenance of nitrogen equilibrium. Albanese (1950a) listed it as probably not required by infants. All of this suggests in the light of our earlier discussion of the gradation of states in which genes may exist that some individuals may possess metabolic machinery which makes the production of arginine difficult. If this is the case such individuals might benefit from arginine administration. Albanese (1950b) cites an example in which a very low sperm count in a 30 year old individual was increased severalfold by the administration of 8 gm of arginine. Since the arginine content of sperm cells is extraordinarily high the finding seems a reasonable one.

The question of the beneficial effects of glutamic acid administration on mentally deficient children has been the subject of a great deal of discussion. That suggestive results (speaking conservatively) have been obtained makes more probable the possibility that in some individuals the metabolic machinery for producing this amino acid has a limiting effect, from the functional viewpoint. The evidence is even stronger that glutamine production may be a limiting factor for maximum health.

in certain individuals. Its efficacy in the treatment of ulcers (Sluue *et al*, 1957) and of alcoholism (Rogers and Pelton 1957a) is strongly indicated and preliminary studies indicate that its administration enhances the test scores of mentally deficient children (Rogers and Pelton 1957b). This is obviously a case where no all-or-none law applies. If there are some individuals who are markedly benefited by glutamine administration and others who are not affected at all, this is what one might expect on genetic grounds.

A general observation which is in line with the concept we are discussing is that the so called nonessential amino acids do not belong as strictly in this category as the first evidence indicated (Albanese 1950c). On the basis of present evidence the possibility exists that the list of amino acids essential for maximum health may not be identical for all individuals.

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## CHAPTER 4

# Utilization of D-Amino Acids

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## I INTRODUCTION

The unique role of dietary protein is to provide the amino acid units needed for the synthesis of new tissue and for the structural repair and proper maintenance of the functioning cell. How well it fulfills this purpose can best be judged by the measurement of its capacity to support growth in the subject to which it is fed to induce depletion after

fasting, or to promote nitrogen retention. The same is true of an amino acid mixture. Actually measurements of this type are largely limited to studies involving the essential amino acid whose exclusion from the diet prevents the organism from responding normally. Hence the comments in this chapter will be limited largely to an examination of the question of how well the D enantiomorphs of the essential or indispensable group of the amino acids may function when they are provided singly or collectively in lieu of their natural L counterparts, or in addition to them.

Rose (1938) has defined an indispensable dietary component as one which cannot be synthesized by the animal organism, out of materials *ordinarily available*, at a speed commensurate with the demands for *normal growth*. Under experimental conditions in which growth is not a suitable index, this definition requires modification. In the adult subject the criterion most commonly employed is maintenance of nitrogen equilibrium. Usually an amino acid which is essential for growth is also essential for maintenance. Arginine is a notable exception probably because it can be synthesized rapidly enough to meet the needs imposed by maintenance but not the additional demands for normal growth (Borman *et al*, 1946). In the human adult subject, both arginine (Rose *et al*, 1954a) and histidine (Rose *et al*, 1951) are dispensable for the maintenance of nitrogen equilibrium. Their requirements in the human infant have not been established.

## II AVAILABILITY OF THE D AMINO ACIDS FOR MAINTENANCE AND GROWTH

Qualitative comparisons of the D and L forms of the essential amino acids indicate that, in some instances both modifications can be used for tissue synthesis or repair, but that in other instances only the L form will suffice for either purpose. Table I summarizes these relationships in the rat, the mouse, and the human subject. It also includes observations on cystine and tyrosine, whose presence in the diet makes the inclusion of less methionine and phenylalanine necessary but it affords no indication as to the relative readiness with which growth is supported or nitrogen equilibrium is maintained.

### A PROMOTION OF GROWTH IN THE RAT

#### 1 Tryptophan

The degree of utilization of the D enantiomorph for growth in the rat as compared with its L counterpart differs widely among the various amino acids. It depends in part upon the rate of growth which the basal diet in which it is being substituted for the L enantiomorph will

support. In the early comparisons of the capacities of D and L tryptophan to induce growth in the rat little or no difference in rate of response or in food consumption was evident when the isomers were fed at levels of 0.2% of the diet. Weight gains on a level of 0.1% were smaller but also about the same for either isomer (Berg 1934). No

TABLE I

UTILIZATION OF THE D-MODIFICATIONS OF CYSTINE, TYROSINE AND THE AMINO ACIDS ESSENTIAL FOR GROWTH IN THE RAT AND MOUSE AND FOR THE MAINTENANCE OF NITROGEN EQUILIBRIUM IN THE ADULT HUMAN SUBJECT

Amino acid	Rat <sup>a</sup>	Mouse <sup>a</sup>	Man <sup>a</sup>
Tryptophan	+ (1 <sup>b</sup> 2 3)	— (4)	— (5 <sup>b</sup> 6 7 <sup>b</sup> )
Lysine	— (8 <sup>b</sup> 9 <sup>b</sup> 10)	— (11)	— (12)
Methionine	+ (13 14)	+ (15)	+ (16)
Threonine	— (17)	— (15)	— (16)
Phenylalanine	+ (18)	+ (15)	+ (19) <sup>c</sup>
Leucine	+ (20)	— (15)	— (21)
Isoleucine	— (22 23)	— (15)	— (21)
Valine	+ (24 <sup>d</sup> 25 26)	— (15)	— (27)
Histidine	+ (28)	— (4)	<sup>e</sup>
Arginine	+ (29)		<sup>e</sup>
Cystine	— (30)		
Tyrosine	+ (31)		

<sup>a</sup> In each column + indicates that at least some utilization was recorded in the reference cited. — indicates that no utilization was observed. Numbers in parentheses refer to bibliographic references listed below the table. Full reference citations are given at the end of the chapter.

<sup>b</sup> In these tests the DL form rather than the D amino acid was used.

<sup>c</sup> Cannot be utilized above a maximal level.

<sup>d</sup> This was a repletion study.

<sup>e</sup> Not essential for maintenance of nitrogen equilibrium.

## REFERENCES TO TABLE I

- |                                    |                                     |
|------------------------------------|-------------------------------------|
| 1 Berg and Potgieter (1931)        | 17 West and Carter (1938)           |
| 2 du Vigneaud <i>et al</i> (1932a) | 18 Rose and Womack (1946)           |
| 3 Berg (1934)                      | 19 Rose <i>et al</i> (1955a)        |
| 4 Celander and Berg (1953)         | 20 Reicheggl <i>et al</i> (1958a)   |
| 5 Albanese <i>et al</i> (1948)     | 21 Rose <i>et al</i> (1955d)        |
| 6 Baldwin and Berg (1949)          | 22 Greenstein <i>et al</i> (1951)   |
| 7 Rose <i>et al</i> (1954b)        | 23 Albanese (1945a)                 |
| 8 McGinty <i>et al</i> (1924 1925) | 24 White <i>et al</i> (1952)        |
| 9 Berg and Dalton (1934)           | 25 Wretling (1956)                  |
| 10 Berg (1936)                     | 26 Womack <i>et al</i> (1957)       |
| 11 Totter and Berg (1939)          | 27 Rose <i>et al</i> (1955e)        |
| 12 Rose <i>et al</i> (1955b)       | 28 Cox and Berg (1934)              |
| 13 Jackson and Block (1937 1938)   | 29 Winitz <i>et al</i> (1957)       |
| 14 Wretling and Rose (1950)        | 30 du Vigneaud <i>et al</i> (1932b) |
| 15 Bauer and Berg (1943)           | 31 Bubl and Butts (1948)            |
| 16 Rose <i>et al</i> (1955c)       |                                     |

essential differences in rates of growth had been observed in similar comparisons with L- and DL tryptophan (Berg and Potgieter 1931), or in tests in which L- and D tryptophan were fed separately (du Vigneaud *et al*, 1932).

In the twenty years which followed basal diets had been formulated which were able to promote much more rapid gains in weight (3.6 vs 1.8 gm per day). Extensive reinvestigation with such diets from which nicotinic acid and liver extract had been omitted (Oesterling and Rose, 1952) indicated that it was now possible to obtain growth at the 0.2% dietary level which was only 93% as rapid in 28 days with D as with L-tryptophan, at the 0.15% level it was only 61% as rapid. In each instance, the diminished rate of growth on D tryptophan was equivalent to that which would have been produced on one fourth less L tryptophan. Factors responsible for the more rapid growth on the newer basal diet can only be surmised from the differences in dietary composition. The yeast and probably also the yeast concentrate employed in the earlier tests undoubtedly furnished small amounts of tryptophan. It must also have been less adequate as a source of the vitamins of the B complex than are the crystalline mixtures which it is now possible to supply. There seems to be little doubt that the considerable reduction in the fat content of the diet (from 22-24% to 2%) also favored the growth response. The improvement observed in the sensitivity of the tests is somewhat analogous to that noted with arginine which can be shown to be an essential amino acid for growth in the rat only when the diet permits relatively rapid growth (Borman *et al* 1946). Earlier investigations had proved beyond doubt that arginine was readily synthesizable in the rat and had seemed to warrant the assumption that in this species arginine was not an essential dietary component (Scully and Rose, 1930). It is now recognized as essential only because its rate of synthesis is not sufficiently rapid to meet the needs of maximal growth.

## 2. Histidine and Methionine

In the comparisons of gains in weight induced in the rat by D vs L histidine it was obvious from the outset that D histidine was the less readily utilisable for growth purposes (Cox and Berg 1934). Of all of the essential amino acids whose D forms can be utilized for growth D methionine seems to replace its natural L counterpart the most efficiently. This was true even at suboptimal levels as low as 0.2% in diets which contained 0.2% of L cystine (Wretling and Rose 1950).

## 3. Phenylalanine

Comparative tests of D and L phenylalanine at levels of 1.0% in high fat diets which contained no tyrosine led Rose and Womack (1946)

to conclude that d(+) phenylalanine induces growth which is almost if not quite as satisfactory as when l(—) phenylalanine is the supplement. Their data do show differences as they themselves noted "It will be observed that the subjects which received the d(+) phenylalanine grew quite satisfactorily although the total gains were not quite so large as in the animals which received the l(—) phenylalanine. Whether the differences are significant is not clear. In a reinvestigation of the problem Armstrong (1953) reports finding much wider divergencies in response to the two isomers. He implies that the better food consumption and the more rapid growth response obtained in his comparisons were attributable to his use of a better diet. This is later given (Armstrong 1955) as the probable reason for the 50% more rapid growth reported on 12% L phenylalanine than on the approximately 0.9% level previously assumed (Rose and Womack 1946) to induce maximum increases in weight. Probably through oversight no suggestion is made as to the nature of the dietary improvement. Both papers (Armstrong 1953 1955) state that the conditions and basal diets were the same as those used previously. The diet described (Armstrong and Lewis 1950) is essentially the same as that of Rose and Womack (1946) except for differences in the composition of the amino acid mixture. Armstrong's technique involved feeding his rats during a preliminary period of 5 days on diets containing tyrosine but not phenylalanine then transferring them to the phenylalanine diets. Such preliminary depletion produces a more immediate growth response to the experimental ration.

#### 4 Valine

White *et al* (1952) subjected rats weighing 110–112 gm to an extensive depletion period of 28 days on a valine free diet which contained an amino acid mixture composed primarily of L amino acids. During a subsequent period usually of 6 days in which the diet was supplemented with 120 mg of D valine daily gains of approximately 1 gm per day occurred. Removal of the valine for an intervening 5-day period produced losses in weight. A following 6 day period of supplementation with 120 mg of DL valine produced gains averaging 2 gm per day. Comparisons with L valine were not run. In the tests 6 gm of food per day were allowed of which the valine supplement thus represented 2%. Since these results were contrary to the conclusions of Rose based on unpublished experiments conducted some twenty years before (Rose 1938) the question was reinvestigated by Womack *et al* (1957). Diets containing 1% of D valine were found incapable of supporting growth in weanling rats but slow growth (0.47 gm per day



in one series 0.91 gm in a second) was obtained when the quantity supplied was raised to 2% of the diet. One per cent of L valine under similar circumstances supported growth approximating 3.9 gm per day. Hence, the capacity of D valine to replace the L isomer is very meager.

### 5 Arginine

The capacity of D arginine to support growth has only recently been investigated. Wintz *et al* (1957) included comparative tests of the isomeric modifications of arginine in a series of quantitative nutritional studies made with water soluble, chemically defined diets in which the 9 other essential amino acids were all incorporated in the L form together with 3 different complements of the nonessential L amino acids. Growth occurred on all three of the diets employed. When L alanine was the only nonessential amino acid fed, incorporation of L arginine in the diet induced only a relatively small growth acceleration. In two other diets in which several nonessential amino acids were provided the acceleration induced was marked. D Arginine was definitely stimulatory in the latter type of diets, but only half as much so as the L isomer. The ratios of average gain in weight to average dietary intake were essentially the same, however, whether the supplementing agent was D or L arginine.

### 6 Leucine

For some time D leucine has been considered to be unavailable for growth (Rose, 1938). Within the past few months Rechcigl *et al* (1958a)<sup>1</sup> have reinvestigated the question and obtained evidence to the contrary. Using diets which provided the essential amino acids and cystine and tyrosine in the proportions in which they are found in the rat carcass with urea and the poorly invertible D components of the DL isoleucine, DL threonine, and DL valine supplied to serve as sources of nitrogen for the synthesis of nonessential amino acids, they observed a growth of 38 gm in 27 days when 0.85% of D leucine was provided as compared with 56 gm with 0.85% of L leucine, 46 gm with 0.425% of L leucine and 52 gm with 0.85% of DL leucine. The stimulation of growth was therefore marked, though less than that induced by half as much of the L isomer. Whether there might have been still greater divergence had a basal diet capable of inducing more rapid growth been employed, can obviously not be answered without further study. Some years earlier Anderson and Nasset (1950) had

<sup>1</sup> We are indebted to Dr. Rechcigl for his courtesy in allowing us to study the manuscripts of these communications prior to their publication.

observed that D-leucine could be utilized to replace part of the L-leucine required for nitrogen equilibrium in the adult rat.

As will be noted later Recheigl *et al* (1958b)<sup>1</sup> have obtained evidence that the norleucine employed in the diet of Fierke and Rose (cf Rose 1938) may have prevented utilization of the D-leucine.

### 7 Isoleucine, Lysine, and Threonine

There is to date no valid evidence that the D forms of lysine, threonine or isoleucine can meet adequately the requirements for moderate growth or even for the maintenance of nitrogen equilibrium.

### 8 Cystine and Tyrosine

Cystine and tyrosine are not considered essential because their dietary need can be met completely by methionine (Womack *et al*, 1937) and phenylalanine (Womack and Rose 1946). Their presence in the diet however definitely decreases the quantity of methionine (Womack and Rose 1941) and phenylalanine (Womack and Rose, 1946) required. Experiments of du Vigneaud *et al* (1932b) conducted before this interrelationship was known showed that D-cystine could not replace L-cystine for purposes of growth. Mesocystine was as effective as an equivalent amount of DL-cystine (Loring *et al* 1933). Tests by Bubl and Butts (1948) showed that when diets otherwise adequate but containing amino acid mixtures with phenylalanine in suboptimal amounts were supplemented with 0.5% of D-tyrosine the growth stimulation was quite as effective as when L-tyrosine was employed.

## B GROWTH PROMOTION IN THE MOUSE

Comparative tests in the mouse of the enantiomorphs of the amino acids required for growth in the rat have yielded results which for the most part seem in essential qualitative agreement with those observed in the rat. The results obtained with tryptophan and histidine are notable exceptions (Celander and Berg 1953). In an earlier series of tests in which the vitamin source was a yeast extract (Totter and Berg 1939) which contained traces of the L forms of these amino acids slow growth occurred when D-tryptophan or D-histidine was incorporated in diets containing D-tryptophan deficient or L-histidine deficient casein hydrolyzate. Rigorous exclusion of the traces of the L isomers of these amino acids by the use of crystalline vitamins prevented growth in tests of D-histidine in an otherwise similar diet or in a diet containing an amino acid mixture devoid of L-histidine. Similar observations were made with D-tryptophan in a diet containing an amino acid mixture from which L-tryptophan was excluded. Traces of L-histidine or L-tryptophan

in such diets seemed to improve the utilization of the D isomers sufficiently to promote slow growth. Why this should be the case is not obvious. When D lysine was tested in diets containing zein fortified with tryptophan, cystine, and histidine, it failed to augment the slow growth observed on the basal diet as did L lysine (Totter and Berg, 1939). In a series of tests involving the use of amino acid mixtures in diets high in fat, no difference was noted whether the diet contained arginine or not (Bruer and Berg, 1943). Excellent growth was obtained on the D forms of methionine and phenylalanine but none on the D isomers of valine, leucine, isoleucine or threonine. D-Threonine failed to prevent the edema noted on the threonine deficient diet. Since slow growth occurred on diets containing mixtures of amino acids limited to the 10 regarded as essential for the rat, none of the other 10 amino acids could have been absolutely indispensable for the moderate growth observed. The evidence obtained in these tests was not sufficiently extensive to warrant reaching any definite conclusion concerning the relative quantitative availabilities of the D- and L-methionine and the D- and L-phenylalanine. The tests of D-valine and D-leucine should obviously be repeated under conditions which have shown these forms to be partially available in the rat. The amino acid mixtures in which each was tested contained the other in the DL form also DL-norleucine.

Review of the earlier work in the rat and mouse and direct comparison of it with the more recent work involving the same species thus make it abundantly clear that the quantitative experimental results obtained depend on the quality of the basal diet employed, probably including such factors as the adequacy of the vitamin supplements and of the salt mixture and the proportion of fat. In certain instances, amino acid balance may also be involved, particularly under conditions approaching deficiency. Results obtained in different laboratories may be affected by differences in strains of animals. However, these are general aspects whose influence is not limited to experiments involving only the D amino acids.

### C. SUPPORT OF NITROGEN EQUILIBRIUM IN THE HUMAN ADULT

In the adult human subject D-methionine was almost, if not quite, as effective as DL-methionine in maintaining nitrogen equilibrium on diets devoid of cystine (Rose *et al.* 1955c), 10 gm per day of either meeting the minimal requirements in the 2 subjects included in the protocols published. It is of some interest to note that 80-89% of the minimal methionine needs could be met by L-cystine (Rose and Wixom, 1955a). In the rat only one sixth of the minimal needs for growth could be similarly satisfied (Womack and Rose, 1941). The only other D-isomer

of an essential amino acid which can be utilized in appreciable degree by the adult human subject for the maintenance of nitrogen equilibrium is D-phenylalanine. In this instance only a limited amount, approximately 0.5 gm per day, appeared to be utilizable under the experimental conditions employed (Rose *et al* 1955a). The estimated minimal daily requirement of 1.1 gm could be met almost as effectively by DL-phenylalanine as by the L isomer but it could not be met fully by D-phenylalanine even though as much as 1.5 gm or 2.2 gm was provided. In a subsequent study inclusion of adequate L-tyrosine in the basic amino acid mixtures was found to exert a sparing effect amounting to 70-75% of the phenylalanine needs (Rose and Womack 1955b). In earlier studies in the rat approximately 50% was thus spared (Womack and Rose 1946). It would be interesting to know whether under these less demanding circumstances man's need for phenylalanine could possibly be completely met by the D isomer.

### III INVERSION OF THE D AMINO ACIDS

Insofar as this writer is aware no protein from an animal tissue has been proved to contain a D-amino acid. It is therefore assumed that before a dietary D-amino acid can be used in the synthesis of new tissue protein or in the replacement of old it must be converted to the L form.

#### A HISTIDINE

The inversion of the D isomer of an essential amino acid was first proved by Conrad and Berg (1937a) who found that the increment of L-histidine in the tissues of young rats fed histidine deficient diets supplemented with D-histidine exceeded the sum of the amount present in the tissues when the feeding began (judged from analyses of sacrificed litter mates) plus the trace amounts of L-histidine present in the basal diet and the vitamin source consumed during the growing period. The histidine was precipitated from acid hydrolyzates of the vitamin source and the carcasses (less the alimentary tract) as the silver salt. An aliquot was employed for colorimetric estimation and the balance from the carcass hydrolyzates was converted to the methyl ester hydrochloride. The specific rotation of the latter indicated that the histidine isolated was essentially optically pure L-histidine. The markedly lower specific rotation of methyl ester hydrochloride prepared from carcasses to which D-histidine had been added before hydrolysis indicated that D-histidine could have been detected had it been present even in relatively minute quantity.

## B TRYPTOPHAN

Kotake and Goto (1937) undertook to prove the "stereonaturalization" of *D* tryptophan by slices and by brei of kidney and liver tissue obtained chiefly from the rat and the mouse, but also from the guinea pig, cat, dog, rabbit, pigeon, and chicken. Production of indole by a strain of *E. coli* able to attack only *L* tryptophan was used as evidence of the inversion. In all species tested the kidney tissue was the more active, the brei more so than the slice. Kidney brei from the mouse was only half as effective as kidney brei from the rat. In the presence of the animal tissue but not in its absence, the *E. coli* produced indole also from indolepyruvic acid, thus suggesting conversion of the latter by the tissue to *L* tryptophan. When *D* tryptophan was fed some indolepyruvic acid was excreted. Small amounts of the *D* amino acid produced a greater keto acid excretion in the mouse than in the rat, with larger amounts the reverse was true but the mouse then also excreted unchanged *D* tryptophan into the urine. Schayer (1950) fed a rat 200 mg of *D* tryptophan which contained 6.08%  $N^{15}$  excess in the indole ring. The rat was sacrificed 48 hours later and the entire carcass, less the contents of the stomach and the intestines, was hydrolyzed with sodium hydroxide in an atmosphere of nitrogen. The acetyl DL tryptophan isolated showed 0.251%  $N^{15}$  excess.

## C LEUCINE

The first use of isotopes to establish incontrovertibly the occurrence of inversion was made by Ratner *et al.* (1940) who added *D* leucine with its carbon chain labeled with deuterium and its amino group with  $N^{15}$ , to the stock diet fed to adult male rats for a period of 3 days. The *L* leucine subsequently isolated from the proteins of the liver and the rest of the carcass contained considerable deuterium but very little  $N^{15}$ . These findings could be accounted for by loss of the original  $N^{15}$  through complete deamination, with subsequent reamination of the keto acid with ammonia from the body's nitrogen pool. On the other hand, isolation of lysine from the tissues of growing rats fed similarly labeled *D* lysine yielded *L* lysine which contained neither deuterium nor  $N^{15}$  (Ratner *et al.* 1943). About half of the *D* lysine was excreted unchanged.

## D VALINE, METHIONINE AND ALANINE

The isolation of *L*-valine which contained  $C^{13}$  from the liver and carcass of rats fed *D* valine containing  $C^{13}$  in its methyl groups has been reported by White *et al.* (1952). Gibson and Smyth (1952) have studied the inversion of  $S^{35}$  labeled *D* methionine by kidney and liver slices from

the rat. The L methionine produced was estimated by converting it to the corresponding  $\alpha$  keto acid with *Neurospora* oxidase and precipitating this as the 2-4 dinitrophenylhydrazone. The radioactivity of the phenyl hydrazone was counted. Corrections were made for the  $\alpha$  keto acid produced from D methionine but not converted to L methionine. Similar tests from the same laboratory with  $C^{14}$  carboxyl labeled D alanine and D leucine have shown the ready synthesis of L alanine by both liver and kidney tissue whereas synthesis of L leucine occurred readily with kidney but only to a small extent with liver (Gibson *et al.* 1954).

#### IV OXIDATIVE DEAMINATION AS AN INVERSION STEP

The mechanism by which stereonaturalization occurs has long been assumed to involve deamination of the D amino acid with loss of asymmetry followed by asymmetric reamination of the  $\alpha$  keto acid thereby produced (Berg and Potgieter 1931). The liver and the kidney of all vertebrates which have been tested contain two different series of enzyme systems for deaminizing the amino acids oxidatively. One series catalyzes the deamination of L amino acids the other that of the D amino acids. This was first demonstrated (Krebs 1935) with tissue slices and crude extracts but the systems involved have since been separated and purified. The general D amino acid oxidases show an absolute stereospecificity. It is clear that they oxidize the different D amino acids at various rates although reports from different laboratories do not agree well in some particulars. This is attributable in part to the various origins of the enzyme preparations employed and to variations in degree of purity. D-Lysine is poorly oxidized if at all and cystine and threonine only to a questionable or minor degree. Tyrosine methionine tryptophan and valine are among the more readily attacked. According to Klein and Handler (1941) phenylalanine isoleucine and leucine are also readily attacked but according to Bender and Krebs (1950) they are less readily oxidized. Histidine (Klein and Handler 1941 Bender and Krebs 1950) and arginine (Klein and Handler 1941) are slowly attacked. The probability therefore seems reasonably good that all of the D amino acids known to undergo inversion (histidine tryptophan methionine phenylalanine valine leucine and arginine) may be converted by D amino acid oxidase to their corresponding  $\alpha$  keto acids as the first step in their inversion. Moreover the readiness with which isoleucine is attacked makes its conversion to the  $\alpha$  keto analog also seem likely.

## V DIETARY REPLACEMENT OF ESSENTIAL AMINO ACIDS BY $\alpha$ KETO ACIDS

The first demonstration that an  $\alpha$  keto acid analog could successfully replace an essential amino acid for purposes of growth was made by Harrow and Sherwin (1926) a little over thirty years ago. How much significance can be attached to the observation that the replacement of histidine by imidazolepyruvic acid resulted in much slower growth than that promoted by L histidine is uncertain because the paper affords no evidence as to the purity of the analog. Imidazolelactic acid was a more effective substitute but imidazoleacrylic (urocanic) acid failed to promote gains in weight in any of the tests recorded. Similar replacement of tryptophan by indolepyruvic acid (Jackson, 1929, Berg *et al.*, 1929-30) induced growth at a rate comparable to that obtained with L tryptophan in equivalent quantity.  $\alpha$  Keto  $\gamma$  methylthiobutyrate is an excellent substitute for DL methionine in the diet of the young rat (Cahill and Rudolph 1942). Replacement of phenylalanine by phenylpyruvic acid has been reported from three different laboratories (Bubl and Butts 1949, Wood *et al.*, 1950, Armstrong and Lewis 1950). Armstrong (1953) notes a more rapid response to phenylpyruvic acid than to D phenylalanine but a less rapid one than to L phenylalanine. *p* Hydroxyphenylpyruvic acid is an excellent substitute for tyrosine (Bubl and Butts, 1949). The conversion of  $\alpha$  ketoisovalerate to valine is reported in a footnote by Rose *et al.* (1942). Wood *et al.* (1950) have observed that it produces growth seemingly superior to that on DL valine, when fed in an amino acid mixture otherwise devoid of valine. Wretling (1952c) also records excellent growth promotion when the  $\alpha$  keto acid of valine is substituted for the amino acid. The comparisons of phenylpyruvic acid and L phenylalanine and of  $\alpha$  ketoisovalerate and DL valine have been verified by Meister and White (1951) who also made comparative tests of leucine and  $\alpha$  ketoisocaproate and of isoleucine and the *d* and *l* forms of  $\alpha$  keto  $\beta$  methylvaleric acid. The growth response to the  $\alpha$  keto analog of leucine was equal to that on L leucine. Isoleucine, like threonine, contains two optical centers, hence exists in four isomeric forms. All four forms were prepared by Greenstein *et al.* (1951) who noted that only one of the four L isoleucine could promote growth in the white rat. D Isoleucine is attacked by an amino acid oxidase to yield L  $\alpha$  keto  $\beta$  methylvaleric acid, the keto analog of L alloisoleucine and D alloisoleucine is attacked by an amino acid oxidase to yield D  $\alpha$  keto  $\beta$  methylvaleric acid, the keto analog of L isoleucine. Meister and White (1951) found the response to the *d* isomer about the same as the response to L isoleucine. The response to the *l* isomer was significantly less. Meister (1954b) was unable to demonstrate that the keto analog

of arginine could accelerate the growth of weanling rats fed a diet free of arginine. More recently, however, this has been accomplished by Winitz *et al* (1957) who found that  $\alpha$ -keto  $\delta$ -glutamic acid plus  $\alpha$ -amino nitrogen in the form of L-alanine would promote extra growth on an arginine free diet though at a rate even less rapid than that induced by D-arginine.

In each of the instances cited a single amino acid was replaced by its  $\alpha$ -keto analog. Wood and Cooley (1954) have conducted tests in which they have replaced simultaneously 5 of the essential amino acids in a mixture of the essential amino acids plus glutamic acid with their corresponding  $\alpha$ -keto acid derivatives. Glycine and 0.2% aspartic acid were added to the diet containing the  $\alpha$ -keto acids to provide nitrogen equivalent to that present in the L-leucine, DL-isoleucine, DL-valine, DL-phenylalanine and DL-methionine replaced. Comparisons showed essentially as rapid growth on the modified diet as on the original one.

There seem to have been no similar studies of the  $\alpha$ -keto analogs of lysine and threonine. The former has been obtained by Meister (1954a) by oxidizing  $\epsilon$ -N-carbobenzoxy-L-lysine with snake venom followed by catalytic hydrogenation to remove the carbobenzoxy group. The keto acid has a pronounced tendency to cyclize to  $\Delta^1$ -piperidine-2-carboxylic acid. Production of L-lysine by the nonenzymatic transamination of the  $\alpha$ -keto acid (or the monohydrate of the cyclized product) with pyridoxamine can be shown by the action of lysine decarboxylase. As indicated earlier, however, when D-lysine which contains stably bound deuterium in its chain and  $N^1$  in its  $\alpha$ -amino group is fed to a young rat the lysine isolated from the tissues contained neither the deuterium nor the  $N^{15}$  (Ratner *et al* 1943). Threonine is the only other amino acid beside lysine which fails to show an excess of  $N^{15}$  when it is isolated from the tissues of the rat fed glycine  $N^{15}$  (Elliott and Neuberger 1950) or  $N^1$ -L-leucine (Meltzer and Sprinson 1952). Like the L-lysine isolated from the tissues of the rat fed deuterio  $N^{15}$ -L-lysine (Weissman and Schoenheimer 1941) the L-threonine isolated after the feeding of  $4\text{-C}^{14}$   $N^{15}$ -L-threonine showed a ratio between the two isotopes nearly equal to that in the doubly labeled amino acid fed (Meltzer and Sprinson 1952). Evidence of this type seems to set lysine and threonine apart as unique essential amino acids whose  $\alpha$ -amino groups are not available for reversible transfer reactions.

To judge from the evidence available to date in the rat the D-isomers of the essential amino acids show a spectrum of invertibility ranging from D-methionine as the most readily inverted amino acid at one end to D-lysine which shows no evidence of any inversion at all at the other end. Near the methionine end lies D-tryptophan. Occupying interme-



dite positions are D histidine, D phenylalanine and D arginine. D Valine is sufficiently readily inverted to promote growth under favorable conditions, as is also D leucine. D Isoleucine is apparently too poorly invertible to promote growth. The application of even such a sensitive criterion as isotope detection fails to show the inversion of D lysine. The degree of invertibility of D threonine has not been similarly tested but the meager lability of the isotopically marked  $\alpha$  amino group of L threonine suggests that little more inversion is likely to occur than in the case of D lysine.

## VI GROWTH RESPONSE ON AMPLE MIXTURES OF THE DL-AMINO ACIDS

Several years ago Van Pilsum and Berg (1950) reported that no appreciable deleterious effect could be detected in 28 days in rats fed mixtures of the DL modifications of the 10 essential amino acids which represented 18.6-21.2% of the diet. This conclusion was based on the observation that animals fed diets containing such mixtures grew as well as animals fed diets containing only the L components, and also on the further observation that additional nitrogen in the form of glycine and diammonium citrate did not enhance the growth on either the L- or the DL mixture. In the initial tests recorded, the L-amino acid mixture fed (11.2%) had contained twice the minimum amounts of each of the essential amino acids which had been tentatively indicated by Rose (1937) to be necessary to support normal growth *when the nonessential amino acids were also supplied*. The doubling was done to compensate for the lack of the nonessential amino acids. Feeding twice this quantity (22.4%) of the same amino acids in the DL form produced a growth retardation and deposition of iron in the spleen which was traced to excessive methionine (2.4%) as the cause. The condition was fully corrected by lowering the methionine intake to 1.2%. Tests of L, DL and D methionine showed that an excess of the natural L form was even more deleterious than an excess of the D isomer. This observation has been confirmed by Wretling and Rose (1950). Wretling (1952b) has since noted that, in excessive amounts (16%), the L isomer of phenylalanine is also the more deleterious. Harper *et al* (1955) have reported that in diets containing only 9% of casein as the chief source of nitrogen, 3% of DL leucine is less toxic than 3% of L leucine in the sense that it depresses growth less. We are inclined to assume that the reason lies in differences in the degree or in the routes of metabolism of the two isomers. No technique has yet been devised however which will satisfactorily rule out differences in food consumption as the probable cause of differences in growth or substantiate beyond doubt the converse.

In experiments of the type cited above the D components of the DL mixture were essentially extraneous. The DL amino acid diets contained enough preformed L amino acids to eliminate any need of having to provide them by inversion. Failure of glycine and diammonium citrate to enhance the growth still further seemed to indicate that at this level the L amino acids as well as the DL mixtures provided adequately for the synthesis of the nonessential amino acids.

## VII TOXICITY OF THE D AMINO ACIDS

The optical isomers of the essential amino acids plus alloisoleucine and allothreonine have been tested for their toxicity in rats when administered intraperitoneally in lethal dosages both individually and in mixtures (Gullino *et al.* 1956). Evaluation of the  $LD_{50}$ ,  $LD_{99}$ , and  $LD_{99.99}$  levels indicated that L and D-alloisoleucine were the least toxic. Most toxic of the L amino acids was L-tryptophan and most toxic of the D-amino acids was D-arginine monohydrochloride. Comparisons at the  $LD_{50}$  level showed that D-tryptophan was only a third as toxic as L-tryptophan and that D-threonine was a little less than half as toxic as L-threonine. In most instances there was less difference. The D-forms of isoleucine, phenylalanine, histidine monohydrochloride, and arginine monohydrochloride had about the same toxicity as their L isomers and the D forms of the other amino acids tested were only slightly less toxic than their L-counterparts. When mixtures of the ten essential amino acids were so compounded that the individual amino acids were present in proportion to their  $LD_{50}$  values there was a "mutually protective effect" i.e. the toxicity was considerably less than the calculated mean value of the  $LD_{50}$ s of the component amino acids. In the L amino acid mixtures L-arginine showed the greatest protective effect. No such protection was afforded by the D-arginine in the D-amino acid mixtures. With L-arginine omitted the "mutually protective effect" was essentially the same for the L as for the D-amino acid mixtures.

Since the protective effect of L-arginine could possibly have been due to its alleviation through urea production of the toxicity of ammonia produced from one or more of the other components of the mixture the capacity of arginine, citrulline, ornithine and several derivatives to prevent death was tested in rats by their injection 1 hour before the injection of an  $LD_{99.99}$  dose of ammonium acetate. At sufficiently high levels the L forms of arginine HCl, citrulline, and ornithine-HCl conferred complete protection. The corresponding D-forms afforded protection to only a fraction of the animals, probably through inversion.  $\alpha$ -keto- $\delta$ -guanidovaleric acid was also somewhat protective (Greenstein *et al.* 1956). Animals injected with  $LD_{99.99}$  levels of L and D-amino acids alone

usually died with elevated blood ammonia and with elevated blood urea. The blood urea was extremely high after the administration of D-leucine and D-lysine HCl, in the latter case nearly as high as that after the administration of D-arginine HCl. L-Tryptophan produced about the same increase in urea as L-arginine HCl. With L-valine and L-and D-histidine HCl the ammonia increased, but the urea did not thus suggesting possible inhibition of the urea synthesizing mechanism (du Ruisseau *et al*, 1956).

Of some interest in connection with the protective effect of D-arginine is the observation made some years ago (Albanese *et al*, 1945b) that arginase extracts from the livers of rats apparently attacked only the L component of DL-arginine but that a similar extract of the human liver was able to produce urea from the racemic form as rapidly as from natural L-arginine. This is in line with the observation of Greenstein *et al* (1956) that in the rat the D-arginine probably becomes effective through inversion.

Injections of LD<sub>50-90</sub> levels of the L- and D-amino acids produced an initial hyperglycemia in varying degree but the animals died with blood sugar values ranging with the amino acid from a marked hypoglycemia to a marked hyperglycemia. Approximately the same patterns were produced with the D-amino acids as with the L-forms. D-Tryptophan was the single exception. Whereas L-tryptophan produced hypoglycemia at death, D-tryptophan induced a marked hyperglycemia. Tests of liver glycogen and urinary glucose indicated that the marked hypoglycemia, characteristically associated with several of the amino acids injected could not be attributed to either glycogen deposition in the liver or to urinary glucose excretion. The decrease probably arose from "utilization" due to violent stress (Winitz *et al* 1956).

## VIII GROWTH RESPONSE ON MARGINAL OR SUBOPTIMAL LEVELS OF DL AND D AMINO ACIDS

### A POORLY INVERTIBLE D AMINO ACIDS AS A NONSPECIFIC SOURCE OF NITROGEN

When ample amounts of only the essential amino acids were provided in the DL-form (18.6-21.2%), as previously noted little or no stimulation of growth was induced by the extraneous D-amino acids in the diet or by the addition of glycine or diammmonium citrate beyond that promoted by the L-amino acids in the mixture (Van Pilsum and Berg 1950). When however the L-amino acids in the mixtures were fed at a lower dietary level (5.9%) as in the study of Phillips and Berg (1954) some evidence was obtained that supplementation with the poorly invertible

D-isomers (of lysine threonine leucine isoleucine and valine) could accelerate the rate of growth beyond that observed on the L amino acids alone although not as markedly as could glycine and diammonium citrate. Some utilization of the extraneous D amino acids under these circumstances therefore seemed likely (probably for nonessential amino acid synthesis or for other purposes more adequately met by glycine and diammonium citrate).

Bimbaum *et al* (1957b) have observed that the addition to their diet of 157 gm of total nitrogen per kilogram in the form of the invertible D arginine HCl or D alanine greatly improved the growth response in 21 days (27.3 gm and 40.2 gm vs 9.0 gm). The unsupplemented diet contained 9.5 gm of total nitrogen per kilogram in the form of the L isomers of the ten essential amino acids. However when the L forms of arginine HCl and alanine were fed singly as supplements under analogous conditions a much greater response was produced (55.5 gm and 61.2 gm) in the same period. Urea supplementation afforded a 24.8 gm growth response, glycine 14.5 gm, ammonium acetate 48.2 gm and ammonium L glutamate 62.2 gm. Some L amino acids (serine hydroxyproline and cysteine) were toxic at the 157 gm total nitrogen level.

#### B READILY INVERTIBLE D AMINO ACIDS FED EN MASSE AS THE SOURCE OF THEIR L ENANTIOMORPHS

When the 2.4% of methionine tryptophan phenylalanine histidine and arginine used by Phillips and Berg (1954) in their 5.9% L amino acid mixture were replaced by 2.4% of the DL modifications slower growth was observed despite the assumption that the D components were as readily invertible as the tests of each singly had previously suggested. Still poorer growth was obtained when this "invertible group" of amino acids was supplied entirely in the D form. Doubling the allotment of the invertible D amino acids failed to enhance the rate of growth perceptibly.

When comparisons were made at a basic L amino acid level representing 2.95% of the diet replacement of the 1.2% of the amino acids belonging to the invertible group by their D counterparts also produced growth retardation. In this instance however doubling the allotment of the invertible D amino acids did accelerate the rate of growth.

A tentative interpretation of this type of evidence which seems feasible is that the capacity of the animal to invert D-amino acids is limited. When the maximal capacity is reached provision of extra D amino acids does not accelerate the rate of growth because no more L amino acids can be produced by stereonaturalization. If on the other hand the quantity of D amino acids provided is much less than the

vertible limit, the quantity inverted, and hence the growth, can be increased by increasing the amount provided

This explanation may be in oversimplification. After all, it is not clear how or to what extent the proportions of the various amino acids needed for the best growth possible at any given dietary level may change with change in the dietary level

Greenstein *et al* (1957) have compared the growth rates obtained on 50% aqueous diets, some of which contained L amino acids exclusively, others DL amino acids. Of the dry weight of one of the L amino acid diets, 62% consisted of the essential amino acids (calculated as free amino acids), with histidine, tryptophan, methionine and phenylalanine in essentially the same proportions as in the 59% essential L amino acid diet used by Phillips and Berg (1954). The diets also contained nonessential amino acids, however, to provide 15.7 gm of nitrogen per kilogram. In the DL amino acid mixtures the histidine, tryptophan, methionine and phenylalanine were fed in the DL form in the same amount as in the L mixture; the arginine and the leucine were fed in the L-form, but all of the other essential amino acids were fed in the DL form at double the L level, the nonessential amino acids, L-alanine, L-serine, and sodium L-aspartate used in the L amino acid diet were replaced with equal amounts of their racemates. Growth on the mixture which contained the DL amino acids was less rapid than on the L mixture. Doubling the DL forms of alanine, serine, and sodium aspartate decreased the growth rate still further. Comparisons of the first two of these diets (Burnbaum *et al*, 1957a) showed ten times as much urinary excretion of  $\alpha$  amino nitrogen on the mixture which contained the DL amino acids as on the L amino acid mixture (52.5 vs 4.6 mg per day) and about twice as much ammonia nitrogen (14.8 mg vs 8.1 mg), but a smaller urea nitrogen output (77.0 vs 124 mg) and a retention by the rat of less total nitrogen (1199 vs 1626 mg). To what extent the limited provision of the DL forms of histidine, tryptophan, methionine, and phenylalanine could have been responsible for these findings one can only guess.

### C INHIBITION OF THE INVERSION OF INDIVIDUAL D AMINO ACIDS BY OTHERS

#### 1 Methionine

Possible interference of  $\alpha$  amino acids with the inversion of another  $\alpha$  amino acid fed at a suboptimal level was first pointed out by Wretling (1952a). He had noted that in a diet which contained no arginine, but all of the other essential amino acids in the DL form, 0.25% of D-methionine produced less rapid growth in 10 days than 0.25% of L-methionine.

(Wretling 1950) At a 1% level however the gains in weight on the two isomers in this period were about the same. When he subsequently fed 0.25% of methionine in a similar diet in which histidine, tryptophan, phenylalanine, lysine and leucine were provided in the L form the rate of growth on the 0.25% of D-methionine was not significantly less rapid than the rate of growth on the 0.25% of L-methionine (Wretling 1952a). Hence he concluded that the stereonaturalization of D-methionine was inhibited either at the decarboxylation or at the reamination stage by the D-forms of some or all of the other amino acids present in the DL-amino acids fed.

## 2 Phenylalanine

Again in an essential amino acid mixture which contained no arginine but all of the other essential amino acids in the DL form Wretling (1952b) observed that suboptimal amounts of the D isomer of phenylalanine (0.5%) also produced significantly poorer growth than the L isomer. Since no comparisons were made with diets containing the amino acids in the L form the probability that inhibition of stereonaturalization was involved can only be inferred. Armstrong (1953) noted a discrepancy at all levels (0.5-4.0%) between the capacities of D- and L-phenylalanine to promote growth on diets allowing more rapid gains in weight. He ascribed the difference in response to differences in food consumption. His diets contained arginine also tyrosine, cystine, glycine and glutamic acid. In Wretling's study the small differences in food consumption were not statistically significant.

## 3 Histidine

We have subsequently undertaken tests of D- and L-histidine in which some of the diets contained all of the other essential amino acids together with 9 nonessential amino acids in the L form and other of the diets contained DL-amino acids (Wachter and Berg 1956). In the L-amino acid diets 0.4% of D-histidine gave an average growth response of 3.4 gm per day vs 4.4 gm when 0.4% of L-histidine was provided. 0.2% of D-histidine produced an average daily gain of 1.2 gm and 0.2% of L-histidine an average daily gain of 4.0 gm. When however the DL-forms of lysine, threonine, leucine and valine and an equal mixture of D-alloisoleucine and L-isoleucine were substituted at twice the L level and DL-alanine and DL-serine at the L level 0.1% of D-histidine promoted an average growth of 1.0 gm per day and L-histidine an average daily growth of 4.2 gm. Decreasing the histidine provided under such circumstances to 0.2% of the diet produced corresponding growth responses of 0.5 gm per day on the D-histidine, 3.8 gm per day on the L-histidine. Compara-

tive growth levels of 4.4 and 4.2 gm per day at the 0.4% L-histidine and 4.0 gm and 3.8 gm at the 0.2% level indicate little interference with growth by the D-forms of the other amino acids when the histidine is provided in the L-form, the similarly compared growth responses of 3.4 gm and 1.0 gm per day at the 0.4% level of D-histidine, and 0.5 gm per day at the 0.2% D-histidine level do seem to indicate so strongly that the presence of the D-forms of the other amino acids markedly inhibits the utilization of the D-histidine.

#### 4. Valine

Perhaps the most striking interference of the D-forms of other amino acids with the utilization of an invertible D-isomer has been noted with valine. Wretling became intrigued with the observation of White (1952) that contrary to the earlier report of Rose (1938), D-valine promoted slow growth. Having observed the retarding effect of the D-forms of several amino acids in the DL-form upon the utilization of D-methionine (Wretling, 1952a) he assumed that the explanation lay in the use of diets consisting mainly of the L-amino acids in one instance and in the use of diets containing several amino acids in the DL-form in the other. In his preliminary report in 1954, he indicated the presence of DL-leucine and DL-isoleucine in a diet which contained both the essential and the nonessential amino acids did markedly reduce the growth-promoting capacity of D-valine. Two years later details of this study were made available (Wretling 1956). Meanwhile we were able to confirm the observation with essential amino acid diets containing 0.7% of L- or D-valine and leucine and isoleucine in the L- or DL-form or the DL-plus-D-forms. In the diet containing all other amino acids in the L-form the D-valine promoted maintenance or very slow growth less than one sixth as rapid as that promoted by L-valine. In the diets containing DL-leucine and DL-isoleucine a slow loss of weight was noted which became somewhat greater upon the further addition of D-leucine and D-isoleucine (Gerulak and Berg 1956).

Wretling's subsequent detailed report (1956) presents data which indicate that rats fed 2% of D-valine in diets in which only the essential amino acids less arginine were provided in the DL-form failed to gain weight. Examination of the protocols reveals that on one of these DL-mixtures which provided DL-isoleucine amounting to 4.0% of the diet, the mean weight averaged 0.6 gm per day for 10 days; on the second mixture in which the DL-isoleucine was lowered to 1.6% of the diet a slow growth of 0.2 gm per day for 14 days was observed. It seems probable that the difference in isoleucine contents could have created enough of a nutritional imbalance such as Benton *et al.* (1956) have reported can be produced

by the addition of isoleucine to low casein diets to have accounted for this difference in response. Wretling had previously obtained growth of  $12 \pm 0.05$  gm per day on diets containing mixtures of all of the essential amino acids in the DL form. Systematic study of the factors which could conceivably have accounted for the differences in response noted when D-valine was fed in the various diets showed that D-leucine was primarily responsible for the growth depression. Diets with 2% of D-valine which permitted an average growth of  $0.7 \pm 0.09$  gm per day for 33 days when L-leucine and L-isoleucine were fed produced little or no change in weight ( $0.0 \pm 0.05$  gm per day) when DL-leucine and L-isoleucine were provided. When L-leucine and DL-isoleucine were fed gains averaging  $0.6 \pm 0.1$  gm per day were observed.

The growth effect of D-valine has also been studied by Womack *et al* (1957). They found that 1% of D-valine was ineffective even in diets which contained L-leucine and the nonessential amino acids (e.g. average weight losses of  $4.7 \pm 1.4$  gm were noted in 28 days vs weight gains of  $11.54 \pm 1.5$  gm when L-valine was provided). With 2% of D-valine growth averaging as much as  $2.56 \pm 1.8$  gm in 28 days was observed as compared with losses averaging  $1.2 \pm 0.8$  gm when the leucine content of the diet was changed from 1.2% of the L form to 2.4% of the DL form.

The maximal gains of 0.91 gm per day on 2% of D-valine obtained by Womack *et al* (1957) is compared with 1.16 gm by White *et al* (1952) and 1.40 gm by Wretling (1956) suggest that the quantitative differences in response may possibly be attributable in part to differences in strain of rats as well as to differences in experimental procedure. The animals used by Womack *et al* (1957) failed to survive periods of valine depletion as extensive as the periods survived by the rats of White *et al* (1952). Both strains manifested the neurological symptoms of valine deficiency (sensitivity to touch and profound lack of coordination in movement) first described by Rose and Eppstein (1939). On the other hand Wretling's (1956) deficient animals were cachectic but showed no neurological abnormality.

As previously noted the  $\alpha$ -keto analog of valine promotes growth comparable to that induced by L-valine when it is added to a valine deficient diet in which the other essential amino acids (except arginine) are supplied in the DL form (Wretling 1952c). Interference with its reamination therefore seems quite unlikely as a contributory factor either in the retardation of growth on D-valine in the presence of D-leucine or in the poor inversion of D-valine under optimal circumstances. If the  $\alpha$ -keto analog is indeed an intermediate in the inversion the presence of the D-leucine is more likely to interfere with its pro-



duction from the D valine presumably by oxidative deamination. We have obtained evidence that such may be the case. ■ Leucine does inhibit the deamination of D valine by ■ amino acid oxidase (Gerulat and Berg 1958). Yoshimoto (1958) has reported that ■ lysine markedly inhibits the oxidation by ■ amino acid oxidase (from hog kidney) of ■ acetyl DL-lysine and of several DL amino acids, notably alanine, phenylalanine, and valine. The inhibition is competitive.

## IX REAMINATION OF ■ KETO ACIDS

As has already been indicated, there seems to be little doubt that an α keto acid, once produced, can be converted effectively to the L amino acid. This is indicated by the readiness with which all single α keto acids tested have served as substitutes for the corresponding L amino acid. It is emphasized even more dramatically in the tests of Wood and Cooley (1954) who showed that methionine, phenylalanine, valine, leucine, and isoleucine could all be replaced simultaneously by their α keto analogs, without markedly slowing the rate of growth. Amination of an α keto acid was first observed by Knoop (1910) who isolated the acetyl derivative of the homolog of phenylalanine, γ phenyl α amino butyric acid from the urine of a dog to which γ phenyl α keto butyric acid had been administered by mouth and subcutaneously. Almost simultaneously, Embden and Schmitz (1910) reported the results of tests in which they were able to isolate amino acids (alanine, phenylalanine, tyrosine, and leucine) from liver perfusates to which the ammonium salt of the corresponding α keto analog had been added. Extensive study of enzymatic transamination has made it clear that this reaction occurs widely and could account for the conversion of the α keto acids to the corresponding L amino acids, or vice versa (cf. Meister, 1955). Recently Radhakrishnan and Meister (1957) have observed that purified L amino acid oxidase from snake venom and D amino acid oxidase from sheep kidney will catalyze the production of L and D amino acid isomers, respectively, under anaerobic conditions from the corresponding α keto acid. Thus L methionine was formed when L leucine was incubated with α keto γ methylthiolbutyric acid and L amino acid oxidase in the presence of flavin adenine dinucleotide. The L leucine was deaminized to produce ammonia for use in the synthesis of the methionine. L Amino acids were not active in the D amino acid oxidase system and vice versa. This may, therefore, represent a pathway, alternate to transamination, for the conversion of an α keto acid to an amino acid.

## X FACTORS AFFECTING THE DEGREE OF AVAILABILITY OF THE D AMINO ACIDS

### A GASTROINTESTINAL ABSORPTION

Evidence is accumulating which suggests that the D isomer of an amino acid may be absorbed at a slower rate than its mirror image, probably as a consequence of the existence of an active process for the absorption of only the latter. Much of the earlier work involved comparisons in the rat by the Cori technique (1925). Usually the L and D isomers were employed and periods of 2-4 hours were allowed. In a few instances some suggestion of relatively minor differences in rate was observed (see Berg 1953). More recently the L and D amino acid oxidases, a transaminase and various L amino acid decarboxylases have been employed in the determination of the relative rates of absorption of the D- or the L-component of a D,L amino acid following its injection into an isolated loop of the small intestine of the adult rat anaesthetized with Nembutal. Where only one isomer was measured by a stereo-specific method the total residuum was determined with chloramine T. With these procedures, Gibson and Wiseman (1951) observed a preferential absorption of the L modification in 0.5-1 hour which was 1.6-8.0 times as rapid as for the D isomer. Of the 13 amino acids employed by them 7 were essential amino acids. A similar observation has been made with D,L histidine and D,L threonine in Thiry Vella loops in unanaesthetized dogs (Clarke *et al.* 1951).

Wiseman (1953) has also reported the use of a circulating device to estimate the ability of the perfused isolated small intestine of the rat to transfer an amino acid against a concentration gradient. Such active transport of the L isomers of alanine, phenylalanine, methionine, histidine and isoleucine, but not of the D isomers could be demonstrated. Agar *et al.* (1954) consider uptake by the cells of the intestinal wall an important step in the transfer. They observed that when isolated washed segments of the small intestine of the rat were placed in a medium containing L histidine the concentration of histidine in the total water of the intestine at equilibrium exceeded that in the surrounding fluid. This was not true when D histidine was employed or when cyanide or dinitrophenol was added to the system. Subsequent study of L histidine absorption from twin loops of rat intestine showed that it was inhibited by L methionine but not by D methionine. The inhibition *in vivo* was much less than had been observed *in vitro* (Hurd and Sidhu 1957). Mathews and Smyth (1954) analyzed the blood stream for L and D enantiomorphs at intervals during the first 25 minutes after the introduction of

mixtures containing only 10% the  $\alpha$  amino nitrogen levels in the plasma were about the same, but 6-10% more acids were excreted into the urine. Van Pilsum and Berg (1950) suggested that the smaller growth retardation produced in rats by an excess of D-methionine may have resulted from its more ready escape into the urine. Crampton and Smyth (1953) have compared the concentrations of the D and L enantiomorphs of alanine, histidine, and methionine in the urine and plasma after injections of the DL mixture into rats. In each instance the concentration of the D modification was lower in the plasma and higher in the urine than that of the L modification. Renal clearance curves for D and L-alanine and D and L-methionine were interpreted as indicating that the L-isomers were reabsorbed by an active stereospecific mechanism in the tubules but that the reabsorption of the D-isomers was due to diffusion alone.

Evidence concerning the excretion of an amino acid or its metabolites after the administration singly of the amino acid obviously cannot provide information by analogy as to its probable utility for the support of nitrogen equilibrium. When it is fed alone no mechanism is provided for its retention, hence its catabolism or its excretion as such becomes inevitable. The situation is analogous to that which obtains when a deficient diet is employed. Schweigert (1947) observed that the rat fed a 12% oxidized casein basal diet fortified with cystine and methionine (leaving the diet deficient in tryptophan) excreted in the free form approximately twice as much of the total histidine, arginine and threonine ingested as the rat fed the same diet supplemented with 0.2% of DL-tryptophan. The amino acids were measured by microbiological assay with *S. faecalis* R. After acid hydrolysis of the urine, the amino acid values showed a two to fourfold increase. Sauberlich *et al* (1948) also noted that both the rat and the mouse excreted more amino acids when the dietary protein was deficient in an essential amino acid than when it was biologically adequate. Moreover, Sauberlich and Salmon (1955) have reported that the tryptophan requirement of the rat is not a constant factor, but is related to the diet employed, especially to the protein or nitrogen level of the diet. The addition of 20% of oxidized casein to a 10% casein diet made it necessary to increase the tryptophan content of the diet from 0.14 to 0.19% to produce comparable growth.

#### D. URINARY EXCRETION OF $\alpha$ KETO ACIDS

In addition to the more ready excretion of the D-amino acids themselves is the possibility that their administration may also induce appreciable  $\alpha$ -keto acid excretion. It was noted earlier that such excretion

was observed by Kotake and Goto (1937) in the mouse and the rat fed  $\alpha$  tryptophan a finding confirmed by others in the rat (Mason and Berg 1952) and in the human subject (Langner and Berg 1955). Several years prior to this Kotake *et al* (1922) had recovered appreciable phenylalanine and phenylpyruvic acid from the urine of rabbits fed 9 gm of  $\alpha$  phenylalanine when a similar dose of L phenylalanine was fed only a trace of the amino acid but more of the keto acid was excreted. DL Phenylalanine produced intermediate outputs of both. Excretion of the  $\alpha$  keto analog of tyrosine was noted after essentially similar tests with 4 gm of L or DL tyrosine (Kotake and Okigawa 1922). Chindler and Lewis (1932) reported a greater excretion of phenylpyruvic acid by rabbits after the administration of DL phenylalanine (4 gm in 48 hours) than after the same amount of L phenylalanine. In his growth studies of D and DL phenylalanine in complete amino acid mixtures Armstrong (1953) observed the excretion in the rat of only a small proportion at high dietary levels none in diets containing 12% of D phenylalanine or 18% of DL phenylalanine. In the apparently normal human subject fed 0.5 gm of D phenylalanine the excretion of phenylalanine and phenylpyruvic acid was fairly widely variable (Gartler and Tashian 1957). Waelsch and Miller (1942) found an increase in  $\alpha$  keto acid excretion by the rat after the administration of the DL forms of several of the amino acids. The kidneys of most mammals contain a higher concentration of D amino acid oxidase per gram of dry weight than does the liver (Krebs 1951). It is quite probable therefore that some of the D amino acids reaching the kidney are converted into their  $\alpha$  keto analogs and ammonia and that both of these are consequently subject to excretion.

Kamin and Handler (1951) have reported that the intravenous infusion into dogs of a mixture of DL amino acids at rates which produced no untoward effects when a casein hydrolyzate was employed raised the ammonia nitrogen of the plasma to levels of 3-4 mg per 100 ml shortly before the death of the animal in 1 hour. As previously indicated Birnbaum *et al* 1957a observed greater outputs of  $\alpha$  amino nitrogen and urinary ammonia but less urea by rats receiving mixtures of essential and nonessential amino acids in which 3 of the nonessential and all of the essential amino acids but leucine were fed in the DL form. The leucine was of the L configuration.

Transaminases are widely distributed in animal tissues. At least some of the several types occur in the kidneys of some animals in concentration as high as in the liver (Cohen 1951). The known activity of these may therefore account for the smaller ammonia production and excretion after the administration of an L amino acid. The transaminases may

been proposed as a possible explanation of incomplete inversion hence poor utilization of certain of the  $\alpha$  amino acids as dietary components. At first thought this may seem quite inconsistent with the observation that several  $\alpha$  keto acids produce growth which is greater than that attainable with the corresponding  $\alpha$  amino acids. But such is not necessarily the case. The situations are not at all analogous. In the actively metabolizing cell in which the  $\alpha$  keto acid is being produced, molecule by molecule, conditions might easily be much more favorable toward its catabolism than toward its asymmetric amination or transamination. *In vivo* evidence in support of such catabolic diversion is, however, very meager and highly circumstantial, at best.

### 1 Valine, Leucine and Isoleucine

We have previously noted that D valine promotes growth much less rapidly when fed at levels approximating 2% of the diet than does the same amount of DL valine (White *et al.*, 1952) or 1% of L valine (Womack *et al.* 1957) but that sodium dimethyl pyruvate equivalent to 2.28% of DL-valine produces a superior response (Wood *et al.*, 1950). D Leucine similarly promotes much less rapid growth than L leucine (Rechcigl *et al.*, 1958a), but its  $\alpha$  keto analog elicits an apparently comparable response (Meister and White, 1951). Of the four stereoisomers of isoleucine, only L-isoleucine supports growth (Greenstein *et al.*, 1951) but the response to D  $\alpha$  keto  $\beta$  methylvaleric acid the keto analog of L isoleucine and D alloisoleucine, is about the same as to L isoleucine (Meister and White, 1951) and a variable response half as great or less, is obtained with L  $\alpha$  keto  $\beta$  methylvaleric acid the keto analog of D isoleucine and L alloisoleucine. In the further catabolism of the keto analogs the branched chain fatty acids produced by decarboxylation and oxidation (isobutyric, isovaleric and  $\alpha$  methylbutyric acid) yield respectively glucogenic, ketogenic and both glucogenic and ketogenic intermediates. The probable mechanisms involved have been relatively recently reinvestigated in tests with the isotopically labeled amino acids or their branched chain fatty acid derivatives and these have been fully verified by studies with heart and liver extracts [for valine and isobutyric acid cf. Fones *et al.* (1951a), Peterson *et al.* (1952) and Robinson *et al.* (1957) for isovaleric acid hence leucine cf. Coon (1950) and Bachhawat *et al.* (1956) and for  $\alpha$  methylbutyric acid hence isoleucine cf. Coon and Abrahamsen (1952), Coon *et al.* (1952) and Robinson *et al.* (1956)].

Some fifty years ago Embden *et al.* (1906) reported that the DL modification of leucine produced considerably more acetone during liver perfusion than did L-leucine. Thirty years later Edson (1935) found

that DL leucine yielded more acetacetate in rat liver slices, and Butts *et al* (1937) found that it produced a greater output of acetone bodies in the fasted rat. On the other hand Hassam and Greenberg (1952) have recently reported a more rapid oxidation of the L form than of the D form of leucine.  $2\text{C}^{14}$  is judged from  $\text{C}^{14}\text{O}_2$  output in 8 hours after the injection of 90 mg of each into 200 gm rats (73.3% oxidation of the L isomer vs 58% oxidation of the D isomer), a smaller urinary excretion of the isotope was observed after the injection of the L form (16 vs 75%).

Edson (1935) had also noted that liver slices from well fed rats produced somewhat more acetoacetic acid from media that contained D isoleucine than from media that contained L leucine or no added leucine. Liver slices from starved rats produced a small decrease in the acetoacetic acid of the medium when L isoleucine was added but little or none upon the addition of D isoleucine.

In the phlorizinized dog a single test of D valine produced a 110% conversion and a test of DL valine a 95% conversion of 3 of the 5 carbons to extra glucose. Under analogous conditions the  $\alpha$  keto analog of valine showed conversions of 101 and 103% and L valine conversions of 46-98% (Rose *et al* 1942). However Fones *et al* (1951b) observed that when 250-300 mg of D valine labeled with  $\text{C}^{13}$  in its methyl groups were fed to fasted rats in equal doses over an 8 hour period slightly over half as much of the administered isotope was recovered in the expired  $\text{CO}_2$  and about half as much in the isolated glycogen as when similar doses of similarly labeled L valine were fed. A much larger proportion of the isotope from the D valine was excreted in the urine (31-34%) than from the L valine (2%).

There is no obvious explanation for the divergence in results noted with differing experimental techniques in the metabolic data cited above for the D and L isomers of the same amino acid. Critical judgment must await the accumulation of additional evidence. The data seem to agree only in their indication that considerable catabolism of the D isomer occurs even when it is administered in relatively large doses.

Though some microorganisms contain racemases able to promote inversion the only avenue of inversion known to date to be available in the mammal is through the readily catabolizable  $\alpha$  keto acid. Once the  $\alpha$  keto acid is produced the course of metabolism is apparently qualitatively the same whether the  $\alpha$  keto acid originated from the L or the D amino acid. The essential difference in availability for protein synthesis is that the L amino acid requires no stereo modification the D amino acid does. Whatever the processes by which the D-amino acid is converted to the  $\alpha$  keto acid and the  $\alpha$  keto acid is converted to the

L amino acid, these must compete with whatever other processes in the living cell may affect either the D or the L amino acid or the intermediates in the inversion

## 2 Methionine and Cystine

Of all of the essential amino acids, methionine alone (in diets containing 0.2% of L cystine) seems to be equally as effective for growth whether fed in the L- or in the D form (Wretling and Rose 1950). D Methionine is also effective as a lipotropic agent (Best and Ridout 1940). Benzoic acid interferes with the activity of D amino acid oxidase in the liver slice, as well as with the transmethylation by D methionine but not by its D keto analog or by L methionine to produce creatine. Hence, stereonaturalization may be prerequisite to the utilization of D methionine for creatine synthesis (Handler and Bernheim 1943). There are striking similarities and differences in the metabolism of D and L cystine (Cavallini *et al.*, 1958). D Cystine cannot replace L cystine in the diet (du Vigneaud *et al.*, 1932b), and mesocystine promotes growth at the same rate as does DL cystine probably because its L cysteine component is available for growth (Loring *et al.* 1933), but its D cysteine component is not. Both D and L cysteine are attacked by liver desulfhydrase to yield hydrogen sulfide, ammonia and pyruvic acid (Fromageot, 1951).

## 3 Threonine

Neither D nor L threonine has been found to undergo appreciable deamination by D or L amino acid oxidase (Krebs, 1951). In the rat however both DL allothreonine and DL threonine increase the deposition of liver glycogen and decrease the ketonuria induced by fasting and butyric acid feeding (Hall *et al.*, 1940 1941). The glycogen production from DL threonine has been confirmed (Hess 1950). There are apparently two routes by which L threonine may be catabolized. One of these involves dehydration and deamination to produce  $\alpha$  ketobutyric acid as first observed with *Bacterium coli* (Chargaff and Sprinson, 1943). In rat liver homogenate, the latter may be aminated to D amino butyric acid (Lien and Greenberg 1953) it may also yield propionic acid. There is no evidence that this reaction is reversible in the animal. Enzyme preparations from the liver and kidney of several species have also been shown to cleave threonine to yield glycine and acetaldehyde (Braunshtein and Vilenkina 1949 Meltzer and Sprinson 1952). Recently Gilbert (1957) has reported that in rat liver this reaction appears to be reversible but that production of allothreonine may have predominated. Appreciable synthesis of L threonine *in vivo* is contraindicated by

its essentiality and its irreplacibility by D threonine or by D or L allothreonine. Whether D threonine is attacked by the enzymes which effect the types of changes indicated with the L isomer is not clear. Lin and Greenberg (1954) found that the D isomers of threonine and allothreonine were not attacked by crude homogenates which yielded glycine and acetaldehyde from the L forms. Meltzer and Sprinson (1952) had earlier suggested that the behavior of DL threonine as a glycogenic and anti-ketogenic agent may be due largely to the D isomer. This view was based on information made available to them privately that L threonine produces only a small rise in urinary glucose in the phlorizinized rat. Swre and Greenberg (1956) note that their purified threonine dehydrogenase does not attack D threonine or DL allothreonine. Nevertheless the results with L threonine suggest possible metabolic routes that may eventually be found to exist also for D threonine. If so the unavailability of D threonine for growth might be ascribed to the essential irreversibility of the metabolic reactions by which D-threonine is degraded.

#### 4 Phenylalanine Tyrosine and Tryptophan

Benzoic acid (Klein and Kamin 1941; Hellerman *et al.* 1946) certain benzoic acid derivatives (Bartlett 1948) and phenylpyruvic acid (Frisell *et al.* 1956) inhibit markedly the activity of renal D amino acid oxidase as do also hydroxybenzoic acid derivatives (Bartlett 1948) and phenylacetic acid to a lesser extent (Frisell *et al.* 1956). Certain indole derivatives are also markedly inhibitory. To secure an optically pure preparation of L phenylalanine in good yield by destroying the D isomer of DL phenylalanine with D amino acid oxidase from hog kidney, Parikh *et al.* (1958) found it feasible to add cyanide to the incubating media to destroy the contaminating catalase and to reincubate the impure end product with fresh D amino acid oxidase again in the presence of cyanide. Similar attempts to prepare the L isomers of tyrosine and tryptophan from their DL modifications, whether in the presence or absence of cyanide, proved impracticable although repeated oxidations did yield products of increasing purity. Inhibition of this sort could conceivably retard inversion in the cell at least temporarily.

When D tryptophan is fed it promotes the production and excretion of D kynurenine in the rabbit (Kotake and Ito 1937) in the rat (Borchers *et al.* 1942; Mason and Berg 1952) and in the human subject (Langner and Berg 1955; Price and Brown 1956). Unlike L kynurenine, D kynurenine yields little or no kynurenic acid in the rabbit (Kotake and Ito 1937). Rat liver slices produce traces of both L and D-kynurenine from D-tryptophan. These are detectable on paper chromatograms and occasionally also a weak spot indicative of kynurenic acid is observed.



After the administration of small doses of DL kynurenine to the rat, no L kynurenine spot could be detected on examining the urine but there was a pronounced D kynurenine spot (Mason and Berg, 1952). As in the rat (Borchers *et al.*, 1942), kynurenine is also excreted in much larger amount in the human subject after the administration of D or DL tryptophan than after the administration of L tryptophan (Price and Brown 1956). Whether such diversion may be of any appreciable significance in nutrition tests with D tryptophan is questionable. It may be important in the rat which utilizes D tryptophan fairly efficiently, but of relatively much less consequence in the human subject and in the mouse which do not. The conversion of tryptophan to kynurenine is irreversible in the rat, L kynurenine being quite unable to replace L tryptophan in the diet (Jackson and Jackson 1932).

## 5 Histidine

Mammalian liver contains an enzyme system which degrades L histidine nonoxidatively to glutamic acid (Edlbacher and Krause, 1930) by a series of irreversible reactions (Tabor, 1955). Without entering into a controversial discussion it may be said that this enzyme system was originally designated histidase and that it is generally considered the dominating system for the catabolism of histidine. Of significance in this consideration are the observations that D histidine is not attacked by most enzymes which attack L histidine (Berg 1953, Tabor, 1955) and that it inhibits the activity of histidase (Edlbacher *et al.*, 1940). D Histidine is only slowly attacked by some D amino acid oxidase systems, more readily by others (Krebs, 1951). It is possible that this may be an important factor in determining the differences in growth response noted in species which require histidine (e.g. in the rat vs the mouse). It seems logical to assume that D histidine promotes glycogen deposition in the liver of the fasted rat (Featherstone and Berg 1942) as a consequence of its initial inversion to L histidine (Conrad and Berg, 1937a) and of the subsequent degradation of the L histidine by histidase. Inhibition of histidase by D histidine may conceivably operate to protect the L histidine produced by inversion in the growing rat from too rapid destruction to permit it to be used for tissue synthesis.

## II Lysine

Neither D nor L lysine has been shown to be readily attacked by mammalian amino acid oxidases (Krebs 1951, Neuburger and Sanger 1944), but D oxidative deamination occurs more readily when the  $\epsilon$  amino group is acetylated (Neuburger and Sanger 1944) or carbobenzyloxyated (Muster 1954a). Advantage has been taken of the reac-

tion of  $\epsilon$ -N carbobenzoxy DL lysine with L amino acid oxidase to prepare D-lysine (Parikh *et al* 1958). The  $\alpha$  keto analog of lysine readily cyclizes (Meister 1954a) when the carbobenzoxy group is removed to yield  $\Delta^1$  piperidine 2-carboxylic acid. It is possible that this ring closure may prevent completion of the inversion of D lysine. Though D lysine is largely excreted unchanged when DL lysine is fed to rats, comparisons with similar tests of L lysine indicate that appreciable catabolism also occurs (Neuberger and Singer 1944).

## 7 Arginine

The  $\alpha$  keto derivative of arginine is apparently not attacked by liver arginase (Meister 1954a). The capacity of arginase to attack D arginine appears to vary with species and experimental conditions (Edlbacher and Zeller 1936; Albanese *et al* 1945b). Relatively little evidence is available concerning the oxidative deamination of D arginine, but tests of DL arginine with crude (Krebs 1935; Klein and Handler 1941) and reconstituted (Klein and Handler 1941) D amino acid oxidase prepared from hog kidney indicate that it undergoes oxidation, as judged from oxygen uptake. Reconstituted D amino acid oxidase from sheep kidney did not attack DL arginine (Karrer and Frank 1940).

The observation that the  $\alpha$  keto analog of arginine is somewhat less effective than D arginine as a dietary component and considerably less so than L arginine (Winitz *et al* 1957) correlates well with data on their roles in urea production. When the  $\alpha$  keto analog was injected 30-120 minutes prior to the administration of an LD<sub>50</sub> dose of ammonium acetate in rats, it protected roughly a third to a fourth of the animals, as did also D arginine monohydrochloride, whereas in similar dosage L arginine monohydrochloride was completely protective. Liver slices prepared from fasted rats, which had been injected an hour previously with L or D arginine monohydrochloride or the  $\alpha$  keto analog, showed accelerated consumption of ammonia and formation of urea upon incubation with ammonium chloride. The acceleration was greater with L arginine monohydrochloride than with the D-isomer or the  $\alpha$  keto analog (Greenstein *et al* 1956). The data cited suggest that the functional unit in each instance is L arginine, into which D arginine and the  $\alpha$  keto analog are inefficiently converted.

## G SPECIES DIFFERENCES

The differences in the availabilities of the D-amino acids for growth and the maintenance of nitrogen balance between the rat and the mouse or the human subject are marked in several instances. Any attempt to account for them must be based ultimately on experimental evidence.

A considerable body of fragmentary information pertaining to the metabolism of the D amino acids in the rat has accumulated. However, with possibly a few exceptions, not enough similar information is available for the mouse man or other animals to permit speculating with any confidence as to what may account for the discrepancies which have been noted in availability for tissue synthesis.

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## CHAPTER 5

# The Efficiency of Utilization of Dietary Proteins

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## I INTRODUCTION

Dietary proteins supply the amino acids for cellular protein synthesis and for other metabolic purposes. Protein synthesis can be correlated with three related activities of the living system: one involving maintenance, another repletion of depleted tissues, and the third the growth of new cellular proteins. Given the necessary constituents in the diet, the efficiency of utilization of dietary proteins for synthesis is primarily a function of four variables which are: the patterns of amino acids provided by the diet, the nitrogen intake, the caloric intake, and the physiological state of the individual. The efficiency of utilization of dietary proteins will be discussed therefore in relation to maintenance, to repletion, and to growth, including in the discussion a consideration of the four variables which have a more or less direct effect upon protein metabolism. Such a discussion will be most meaningful, however, if it is developed from the concept of the dynamic state of protein metabolism.

## II THE DYNAMIC STATE OF PROTEIN METABOLISM

Amino acids may enter into one of two metabolic pathways which have been called anabolism and catabolism. Through anabolism, for example amino acids are organized into cellular proteins. Catabolism is the reverse process, involving the hydrolysis of proteins into amino acids which, in turn may be broken down into still simpler products, some of them formed irreversibly and excreted as waste products. Thus the tissue proteins are, to a varying degree, in a dynamic state (Whipple 1948) contributing to and taking amino acids from an over all metabolic pool. This concept of a pool has a certain experimental reality (Rittenberg 1948 1949) which will be developed throughout this discussion of the efficiency of utilization of dietary proteins. The pool, however, should not be considered as a disorganized fluid component of the body but rather as a mechanism for transfer of amino acids from one tissue to another. The data demonstrate also that some tissues are highly labile entering into and drawing from the pool continually, others are less active in the dynamic state while still others are "one way streets, being essentially irreversibly formed, thereby taking from but contributing little to the pool" (Greenberg and Winnick, 1948).

### A BODY PROTEIN STORES" OR RESERVES

Some body proteins particularly plasma albumin and many cytoplasmic proteins of the soft tissues rise or fall in amount as the dietary protein intake increases or decreases. These tissue proteins have been called the labile protein reserves or protein stores (Whipple, 1948). Such a dynamic state can result therefore, in a shift in amino acids from one tissue protein to another through the "metabolic pool". This process of tissue catabolism and resynthesis also leads to an irreversible breakdown of some of the amino acids to yield energy to the living system, urea being one of the end products of this irreversible catabolism. The amino acids so catabolized must be replaced by the diet or synthesized from dietary constituents. If dietary protein intake is restricted, the continual catabolic activity of the living system results in a reduction in amino acid supplied to the "metabolic pool" thereby decreasing the "protein stores" (Allison 1953a).

### B ENDOGENOUS METABOLISM

The variation in excretion of urinary nitrogen with the magnitude of the "protein stores" is illustrated in Fig. 1. These data were obtained while feeding a protein free diet to adult dogs first while they were in a so called normal state second, while they were depleted in "protein stores" and third, after they had been repleted in those stores.

Since all of the nitrogen excreted during these periods of feeding a protein free diet came from body cellular metabolism this urinary nitrogen can be considered to be endogenous in origin. These shifts in excretion of endogenous nitrogen are primarily the result of variations in catabolism of amino acids in that part of the "metabolic pool" which contributes to the formation of urea. Change in urea nitrogen excretion however can be of body nitrogen origin (endogenous) or of food nitrogen (exogenous) origin and be associated with a number of metabolic

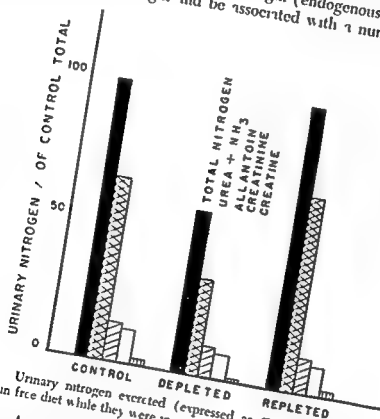


FIG. 1 Urinary nitrogen excreted (expressed as % of control) in adult dogs fed a protein free diet while they were in normal depleted and repleted states

pathways. An interesting shift for example in amino acids from the site of formation of urea to muscle cells may be associated with carbohydrate metabolism. To quote Munro (1956) "After giving carbohydrate amino acids are preferentially deposited in muscle and this causes a reduction in their level in the blood. The amino acid supply to other tissues is thus curtailed with two consequences. First urea production by the liver is reduced so that a fall in urinary output occurs and secondly the tissues other than muscle have a reduced supply of amino acids for synthesis. This later is reflected in the slightly reduced protein content of the liver." Munro suggests that these responses to carbohydrate metabolism are the result of insulin called forth by incoming

ing dietary carbohydrate. Other shifts in hormonal balance alter the interrelationship between anabolism and catabolism and increase or decrease the excretion of urea depending upon the pathways involved in the response (Leatham, 1958). The variable excretion of urea, associated with amino acid deficiencies, amino acid imbalances or other alterations in dietary amino acid patterns is considered in detail later. The general conclusion can be stated, however, that amino acids not used for anabolic purposes, may enter the urea forming metabolic cycle so that the excretion of urea will rise or fall not only with changes in cellular protein (endogenous) but also with dietary protein (exogenous) metabolism. The excretion of urea, on the other hand, of compounds containing nitrogen such as creatinine are approximately constant (see Fig. 1) and independent of cat- abolism of amino acids which enter the urea cycle. Folin (1905), for that matter, emphasized the endogenous origin of creatinine.

### C. NITROGEN BALANCE

This dynamic catabolic, anabolic interrelationship can be introduced into a nitrogen balance which is defined by Eq. (1)

$$B = I - (U + F)$$

where  $B$  is nitrogen balance,  $I$  is nitrogen intake,  $U$  is the excretion of urinary nitrogen, and  $F$ , the excretion of fecal nitrogen. Nitrogen balance therefore is the difference between nitrogen intake and nitrogen excreted. If nitrogen balance is positive, the living system is gaining in protein, either through the growth of new tissues or the repletion of depleted stores. Negative nitrogen balance means a loss in tissue nitrogen through excess catabolism, the dietary protein being insufficient to meet the needs for maintenance of the body tissues. When the intake  $I$  just equals excretion ( $U + F$ ) the system is in equilibrium and is said to be maintaining the status quo condition, activity being nicely balanced with anabolism.

Nitrogen balance, however, is the integrated sum of the gains and losses involving all the tissue proteins of the body. It is possible to have such a dynamic state to have the system go into positive balance, still have some tissues losing nitrogen. Conversely, the system may go into negative balance but some individual tissues may still be in balance. Since urea nitrogen excretion can reflect so many different changes in metabolic pathways associated with both exogenous and endogenous metabolism including the effects of responses of the endocrine system, nitrogen balance responds to many variables. In the following discussion will emphasize a consideration not only

direction but also the rate of change of nitrogen balance as an indication of shifts in nitrogen metabolism within the body of an animal

### III MAINTENANCE OF NITROGEN EQUILIBRIUM

The effect of nitrogen intakes upon nitrogen balance is illustrated in Fig 2. These curves were obtained while feeding different dietary protein sources at various nitrogen intakes over short periods of time to adult dogs so that the protein stores and overall tissue catabolic activity would be essentially constant. The lower curves in negative

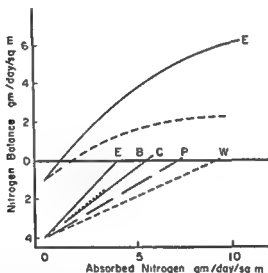


FIG 2 Lower curves illustrate nitrogen balances produced while feeding (over short periods of time) egg proteins E, beef B, casein C, peanut flour P, and wheat gluten W. Upper curves illustrate type of data while feeding a protein of high nutritive value (E) and low nutritive value (dashes) to protein depleted dogs (taken from Allison, 1957).

balance illustrate the type of data obtained while feeding dogs with full "protein stores" and maximum excretion of urinary nitrogen. The dietary protein sources are indicated by the letters at the ends of the curves where E represents egg, B beef, C casein, P peanut flour, W wheat gluten. These curves do not extend into positive balance because the stores of these adult dogs were filled and nitrogen retention, represented by positive balance, could not take place. The upper curve extending into positive balance and ending at E illustrates the type of data obtained while feeding different amounts of egg protein to an individual depleted in "protein stores." The dashed line also extending into positive balance illustrates the type of data obtained while feeding a protein of low nutritive value to a protein depleted animal. The stores



cannot be filled as rapidly with protein of low nutritive value as can be developed with the protein of higher nutritive value. In general, the greater the degree of positive balance that can be produced by feeding a given protein to an adult, the greater the degree of depletion in body proteins. This problem of repletion will be discussed in more detail later but the data in Fig. 2 emphasize the decreased excretion of nitrogen associated with the depleted state and also the relatively small amount of nitrogen required to establish nitrogen equilibrium in the depleted animal. The amount of nitrogen necessary to maintain nitrogen equilibrium in an adult dog depending upon the magnitude of the protein stores can vary from approximately 1 gm per day to over 4 gm per day per square meter of body surface area. Possibly the minimum excretion of body nitrogen would be equal to approximately 2 mg of nitrogen per basal calorie in a normal individual. Such a minimum is supported by the work of Terroine (1936), Smuts (1935), Mitchell (1929), and Bricker *et al* (1945).

### A. NITROGEN BALANCE INDEXES

The curves in Fig. 2 have been represented as linear in the region of negative and low positive balance becoming curvilinear as they are extended into positive balance. Experimentally, the curves are not always found to be linear over so much of the range of nitrogen balance but regardless of the shape, the curves represent the rate of nitrogen retention with respect to nitrogen intake. The tangent of the curve at any intake therefore has been called the nitrogen balance index of that intake (Allison *et al*, 1946). The curves also can be considered as essentially semilogarithmic over the whole range of intakes, thereby expressing the general observation which can be called the law of diminishing returns. This concept applied to protein metabolism, has been emphasized by Almquist (1954) and developed still further by Flodin (1957). Thus the intake producing maximum nitrogen balance would fill the protein stores of the body most rapidly.

### B. BIOLOGICAL VALUES

The nitrogen balance indexes of dietary proteins, illustrated in Fig. 2, represent the fraction of absorbed nitrogen retained in the body of the animal provided the excretion of endogenous nitrogen represented by zero nitrogen intake is constant and independent of nitrogen intake. The fraction of absorbed nitrogen retained in the body has been defined as the biological value of the protein a concept that has been so well developed by Mitchell and his associates (Bricker *et al* 1945). The biological values or indexes for nitrogen equilibrium when the stores

were full were egg 1.14 beef 0.77 casein 0.73 peanut flour 0.56 and wheat gluten 0.44 (Allison 1955)

The data illustrated in Fig 3 demonstrate the adaptive characteristics of nitrogen balances together with the rather constant index obtained during this adaptation. The white bars record the negative nitrogen balance associated with periods of feeding a protein free diet to adult dogs. The bars with slanted lines record the nitrogen balances obtained while feeding a constant amount of wheat gluten nitrogen in alternate periods to those involving feeding of the protein free diet. The first white bar is sufficiently negative to express a catabolic activity of

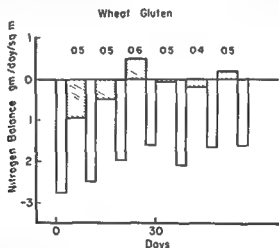


FIG 3 Effect of feeding a protein free diet (white bars) alternately with wheat gluten nitrogen (bars with slanted lines). Figures above bars are indexes (taken from Allison 1953b)

endogenous metabolism that is within the so called normal range. The wheat gluten nitrogen intake was not sufficient to produce nitrogen equilibrium under these conditions. The negative balance associated with both the protein free and nitrogen feeding periods resulted in a reduction in the excretion of nitrogen so that the third period of feeding wheat gluten produced a positive balance. From then on the animals were close to nitrogen equilibrium during the periods of feeding wheat gluten, an adaptation which is illustrated also by the reduced excretion of nitrogen during the periods of feeding the protein free diet. The indexes presented above each period of nitrogen feeding were calculated from Eq (2)

$$\text{Index} = \frac{B - B_0}{I} \quad (2)$$

where  $B$  is the nitrogen balance produced by the nitrogen intake ( $I$ ) and  $B_0$  is the nitrogen balance established during associated periods of

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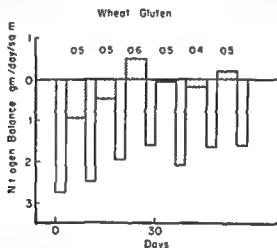


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where  $B$  is the nitrogen balance produced by the nitrogen intake ( $I$ ) and  $B_0$  is the nitrogen balance established during associated periods of

feeding the protein free diet. The tendency for the index to increase when the animal has been depleted may account for the rise to 0 during the third period of feeding the wheat gluten. Certainly in the depleted animal the indexes increased quite markedly in value. In the depleted dog for example, the index for egg was still close to unity (1.12) but the index for casein had increased to 0.84 and the index for wheat gluten was raised most markedly to 0.74 (Allison, 1949). These higher indexes for proteins fed to the depleted animal indicate that potential anabolic processes are raised. Data have been obtained, for example, to demonstrate an increased uptake of  $S^{35}$  labeled methionine in many of the tissue proteins in the dynamic state of the depleted animal (Allison *et al.*, 1958b).

### C. ADAPTATION OF NITROGEN BALANCE

The data in Fig. 3 illustrate one kind of adaptation of nitrogen balance to shifts in catabolic, anabolic interrelationships. Adaptation of the living system to changing conditions of both exogenous and endogenous origins are often reflected by alterations in nitrogen balance and interpretations of the significance of any particular balance should take into consideration adaptive processes. For example, in a growing individual, the balance should be positive and essentially constant while feeding intakes of dietary protein sufficient to establish the growth response. If however, a change in diet is made and the individual goes into positive balance for a short period of time and then drifts into a more negative balance this adaptive procedure may indicate a deficient amino acid pattern. The best balance of amino acids will produce an adequate positive balance and return that balance for optimum growth. Thus the direction of change in nitrogen balance, representing adaptation, can reveal additional information concerning the significance of the response. In many experimental designs these adaptive processes can be very rapid. Another type of adaptive mechanism may represent a shift in body fluids and in the excretion of urea nitrogen. Mention has already been made to the shifts in urea nitrogen formation and excretion that are associated with incoming carbohydrate. It is possible for an initial change in nitrogen balance to represent an alteration in excretion of urea from the body pool rather than in nitrogen retention. An individual for example could go into a negative nitrogen balance in response to a change to a lower intake of a better quality protein even though the body tissues may be in positive balance. The negative balance would be the result of excretion of the relatively excess urea nitrogen formed during the previous period of feeding the higher intake of a protein of low nutritive value. The negative balance just described

however should be followed by a rapid shift into more positive balance in the presence of the protein of high nutritive value

#### D CORRELATION WITH AMINO ACID PATTERN

The magnitude of the balances also can be correlated with the pattern of essential amino acids provided by the dietary proteins to the body, essential amino acids being defined by Rose (1936) as those which cannot be synthesized by the body from dietary sources in adequate amounts to meet the metabolic needs. Essential amino acids must therefore be provided in the daily diet in sufficient quantities and in proper proportions to meet the demands for synthesis as well as for other special metabolic roles. Alteration of the pattern of essential amino acids in the dietary protein can change markedly the retention of dietary nitrogen in the body. For example, if lysine is added to wheat gluten the index can be increased to 0.8. Adding methionine to casein increased the index to values equal to those found for egg proteins.

The nitrogen balance index of unity obtained for egg proteins has been interpreted to mean that the pattern of essential amino acids provided by these proteins is ideal for protein metabolism. All of the dietary nitrogen may be considered to be retained in the body of the animal for purposes of synthesis. Thus the pattern of essential amino acids in egg protein has been used as a reference for the calculation of the nutritive value of other dietary proteins. Mitchell and Block (1946) and Block and Mitchell (1946-1947) using egg protein with a "biological value" of 100 compared the percentage of each essential amino acid to that found in egg protein. A "protein score" was calculated to be the relative amount of the amino acid showing the largest deficit to the corresponding value in egg protein. These "protein scores" were highly correlated with the "biological values" determined experimentally. Oser (1951) sought to improve this type of calculation by taking into consideration through a geometric mean the pattern of essential amino acids thereby expressing nutritive value in terms of an Essential Amino Acid Index. Mitchell (1954) calculated a "modified amino acid index" as the geometric mean of the relative deficits of all the essential amino acids present in smaller quantities than occurs in egg proteins. The correlation between this calculation and experimentally determined "biological values" suggested that the single amino acid most limiting may not entirely determine the nutritive value but that several deficits may contribute to the establishment of this value.

The estimation therefore of nutritive values through a search for the most limiting amount of each essential amino acid needed for maintenance, repletion or growth is a most important feature of the study

of protein nutrition: Such minima have been sought through the use of mixtures of amino acids. A reference pattern of amino acid, for example, has been developed by the Committee on Protein Requirements of the Food and Agricultural Organization (Protein Requirements, 1957) from studies of amino acid requirements for maintenance and growth in man (Rose 1957, Leverton *et al*, 1956, Swendseid *et al*, 1956, Holt and Snyderman, 1956, Jones *et al*, 1956). This pattern, expressed as milligrams of amino acid per gram of nitrogen is isoleucine, 270, leucine, 306, lysine, 270, phenylalanine, 180, tyrosine, 180, total sulfur amino acids, 270, methionine, 144, threonine, 180, valine 270, tryptophan 90.

It was hoped that such a group of minimum requirements would be helpful in determining essential amino acid deficiencies in dietary proteins. These requirements could vary with the age and physiological state of the individual but, for a first approximation, one provisional pattern was suggested to estimate deficiencies. The pattern was called provisional to emphasize the need to determine more accurately the absolute requirements of amino acids and the ratios between them. Obviously shifts in relative rates of absorption of amino acids resulting from delayed digestion or from other causes would also alter the significance of a fixed reference pattern. Analysis of the data in the literature, however, demonstrate that this provisional pattern is very useful in estimating essential amino acid deficiencies and "Biological Values" of dietary proteins.

Although this pattern for amino acid requirements represents a first approximation based upon amino acid studies in man, the pattern eventually developed for protein anabolism will probably be quite similar for most animals. The nitrogen balance indexes of dietary proteins for the rat for the dog, and for man are similar (Allison 1957) except that the rat and the dog require a higher sulfur amino acid intake than man (Cox *et al* 1947) and the rat requires less lysine for maintenance (Mitchell 1947). Other exceptions occur when large amounts of proteins of unusual composition must be synthesized such as wool and feathers. The chick lacks ability to synthesize sufficient arginine and glycine to meet the needs for protein metabolism. The data presented by Nasset (1957) also emphasize the similarity of the essential amino acid requirements for rat and man when placed upon the basis of metabolic body size. These experiments involving minimum requirements of essential amino acids suggest that when nitrogen needs are met, excesses of some of the essential amino acids may occur in proteins of high nutritive value. If excesses do exist, they would contribute to the need for so called nonessential amino nitrogen but they also could at times represent safety factors to control the intake of essential amino

acids under various physiological conditions. This report emphasizes the large number of variables that can effect the utilization of amino acids, one of the most important of these variables being the rate of absorption of one amino acid with respect to another thereby controlling the actual pattern presented to the body. The value however of a reference pattern of minimum requirements of essential amino acids, such as the one proposed by FAO for estimation of dietary deficiencies in these acids cannot be overemphasized. The preliminary studies by Scrimshaw and associates (1957) illustrate the usefulness of the reference pattern to researches on amino acid deficiencies and supplementation in children suffering from protein malnutrition.

#### IV REPLETION OF PROTEIN STORES

The interrelationship between patterns of amino acids provided by the diet, the nitrogen intake and the caloric intake can be illustrated nicely by the effects of these variables upon repletion of depleted "protein stores" in the adult dog. The animals were depleted by feeding a protein free diet for 4 weeks (Allison and Wannemacher 1957) and then repleted by feeding the same diet with constant protein and caloric intakes until nitrogen equilibrium was reached.

##### A THE NITROGEN BALANCE OF REPLETION

The positive nitrogen balance produced in the depleted dog by feeding protein was a function of the nitrogen intake, the caloric intake and the pattern of amino acids provided by the dietary protein.

##### 1 The Effect of Nitrogen Intake

The circles with horizontal bars in Fig. 4 (Allison 1958, b) record the nitrogen balances produced in depleted dogs by repleting with 0.6 gm. of casein nitrogen or of wheat gluten nitrogen per day per kilogram of body weight. The slopes of the lines record the rate of repletion; the areas under the lines represent the amount of dietary nitrogen retained in the body. The caloric intake was maintained constant at 75 calories per day per kilogram of body weight, a caloric intake that is considered adequate for the normal adult animal kept under laboratory conditions. Increasing the nitrogen intake to 1 gm. per day per kilogram of body weight and keeping the caloric intake at 75 calories resulted in a greater nitrogen balance and amount of repletion but the slopes of the repletion lines (illustrated by the open circles) were not altered from the slopes observed at the lower nitrogen intakes (0.6 gm. per day per kilogram N.W.).





wheat gluten nitrogen per day per kilogram of body weight resulted in an initial nitrogen balance of 0.14 the slope was 0.003 and 2.9 gm of nitrogen per kilogram of body weight was retained during repletion. Approximately 1 gm of casein nitrogen per day per kilogram of body weight increased the initial balance to 0.75 the slope to 0.003 and the amount of repletion at equilibrium to 9.5 gm of nitrogen per kilogram of body weight. Supplementing the 1 gm of casein nitrogen with methionine raised the initial nitrogen balance to 0.84 the slope to 0.015 but the amount of repletion when equilibrium was reached was still 9.5 gm of nitrogen per kilogram of body weight. Feeding approximately 1 gm of wheat gluten nitrogen per day per kilogram of body weight resulted in an initial nitrogen balance of 0.63 the slope of repletion line was 0.019 but the amount of repletion when nitrogen equilibrium was re-established was approximately the same as that reached by feeding casein. Supplementing the wheat gluten with lysine increased the initial nitrogen balance to 0.72 and the slope of repletion to 0.034 making the repletion with the supplemented wheat gluten the same as was obtained with casein. These data on repletion suggest that if unsupplemented wheat gluten is fed for a sufficient length of time to reach equilibrium the adult "stores" can be filled with nitrogen in amounts equivalent to those retained during repletion with casein. Further studies are needed however to determine the effect of high intake of a deficient protein upon the balance between various tissue proteins and fat stores. Evidence is being obtained to demonstrate an imbalance of repletion associated with deficient amino acids particularly if the nitrogen balance index is less than 0.6. Such imbalances will be illustrated in part with the data involving the effects of the pattern of amino acids upon growth in young animals. The effect of dietary patterns upon the rates of formation of individual tissue proteins can be illustrated also by the following data on repletion of plasma proteins.

## II. REPLETION OF PLASMA PROTEINS

The black bar in Fig. 5 records the average plasma albumin/globulin ratio in dogs after having been fed a protein free diet for a period of 4 weeks. The dogs were repleted over a period of 4 weeks by feeding different amounts of casein sometimes supplemented with other amino acids or wheat gluten with or without lysine. Feeding 0.2 gm of casein nitrogen per day per kilogram of body weight to these depleted dogs produced only a slight positive balance so that they were kept in a depleted state and the albumin/globulin ratio remained at a low level (illustrated by the bar with crossed lines over casein). Increasing the intake to 0.6 gm of casein nitrogen per day per kilogram of body weight

did not fill the protein stores completely but the albumin/globulin ratio was raised to approximately 0.5, as recorded by the bar with slanted lines over casein. Feeding 1 gm of casein nitrogen per day per kilogram of body weight to the depleted animals over a period of 4 weeks filled the protein stores but did not raise the albumin/globulin ratio (illustrated by the white bar over casein) above the value obtained while feeding the lower nitrogen intake. The development of a low albumin/globulin ratio even in the presence of relatively full protein stores is characteristic of repletion with casein (Seeley, 1945, Chow *et al.* 1948).

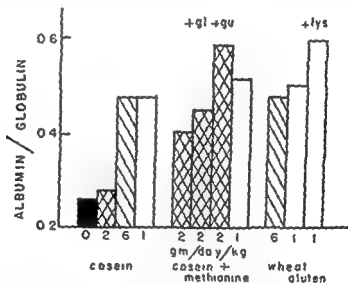


FIG. 5. Albumin/globulin ratios produced during repletion with different amounts of casein and wheat gluten nitrogen fed to protein depleted dogs (see text for details) (taken from Allison and Wannemacher 1957).

The bars over wheat gluten in Fig. 5 demonstrate the more marked effect that this dietary protein has upon the albumin/globulin ratio especially when the gluten is supplemented with lysine. A similar response to the synthesis of plasma albumin in growing rats fed wheat gluten will be recorded later in this report. The formation of plasma albumin was increased by supplementing the casein with methionine, illustrated by the bars over casein plus methionine. Even when the intake was only 0.2 gm of casein nitrogen per day per kilogram of body weight and repletion of body protein stores was quite limited the albumin/globulin ratio was increased markedly by supplementing the casein with methionine. Adding more nitrogen in the form of glycine with the methionine did not increase the ratio above the value recorded for dogs fed casein supplemented with methionine alone. Supplementing the low casein diet however with methionine plus guanidinoacetic acid increased the ratio to normal values even in dogs that were still rela-

tively low in "protein stores" (Allison 1956 Allison and Wannemacher 1957) This response to the addition of guanidinocetic acid with methionine to casein was observed only in animals that had been depleted in one way or another in "protein stores" These results suggest the possibility of altering the rates of repletion of specific tissue proteins possibly for therapeutic purposes by using various mixtures of dietary amino acids (Whipple *et al* 1946 Sebrell and McDaniell 1952) These data also re-emphasize the possibilities that some tissue proteins may be in more positive balance than others during a process of repletion The best over all pattern of amino acids could be the one that promoted the repletion of old or the growth of new tissues in rates balanced most advantageous to the development of some particular physiological state

## V GROWTH AS A MEASURE OF NUTRITIVE VALUE OF DIETARY PROTEINS

Gain in body weight is a measure of the nutritive value of dietary proteins has been most popular Osborne and associates (1919) developed the concept of protein efficiency which has been defined as the grams gained in body weight per gram of nitrogen or protein intake

### A THE PROTEIN EFFICIENCY RATIO AND THE NITROGEN GROWTH INDEX

The variations in protein efficiency for casein for cottonseed meal and for wheat gluten with nitrogen intake in rats are illustrated in Fig 6 These data were obtained by feeding these proteins in a purified diet to female rats from weaning over a period of 28 days (Allison *et al* 1958a) The nitrogen intake then represents the total intake over this 4 week experimental period Osborne and associates recommended that the nutritive value of dietary proteins be expressed as the maximum ratio a recommendation emphasized also by Barnes and Bosshardt (1946) The rise and fall of the protein efficiency ratio with nitrogen intake is the result of the shape of the body weight gain curves illustrated in the lower part of Fig 6 The open circles record the body weight gains of the female rats fed different amounts of casein over the 4 week feeding period The triangles demonstrate similar data recorded while feeding groups of female rats different intakes of cottonseed meal protein The crosses illustrate data obtained while feeding increasing intakes of wheat gluten nitrogen The curves do not pass through the origin since a certain amount of nitrogen is necessary for maintenance of body weight The quantity of nitrogen needed for maintenance was a large fraction of the total intake when nitrogen was restricted so that a protein efficiency ratio started low and then rose to a maximum as the nitrogen intake and body weight gain increased As soon as max

imum gain in body weight had been reached, increasing the nitrogen intakes resulted in a decreasing protein efficiency (Sure, 1957)

The rate of increase in body weight with respect to nitrogen intake, was essentially linear at the lower nitrogen intakes so that the slopes of the curves, correlating body weight with nitrogen intakes, are a good measure of the nutritive value of the dietary protein. These slopes may be called nitrogen growth indexes. Such indexes are of particular value in the rat because growth in this animal is highly correlated with nitro

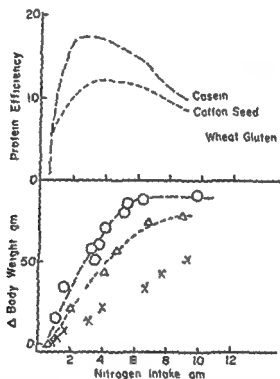


FIG 1 Data resulting from feeding different amounts of casein cottonseed meal or wheat gluten nitrogen to female rats from weaning over a period of 4 weeks

gen retention (Hegsted and Worcester, 1947). The index for the linear portion of the casein curve is 19 for cottonseed meal, the index is 14 and for wheat gluten the index is 6. Supplementing the casein with methionine increased the growth index to 35. A similar experiment involving male rats resulted in the following growth indexes: egg 35, casein 22, cottonseed meal 14, wheat gluten 7. Supplementing casein with methionine increased the index for casein to be equivalent to egg and adding lysine to wheat gluten raised the index toward the value for casein (Allison *et al.* 1958a). There is need however, to determine the optimum amounts of these amino acids needed for supplementation to produce specific effects at low and high nitrogen and calorie intakes. Such a study involving methionine and lysine supplementation has been

reported by Rosenberg (1957) who found that methionine and lysine requirements increased with the energy content of the diet data which emphasizes again the importance of caloric intakes upon nitrogen retention (see also Bressani and Mertz 1956)

## B PLASMA PROTEINS

The effects of increasing nitrogen intake upon the formation of plasma proteins are illustrated by the data recorded in Table I. These data demonstrate the tendency for the development of a rela-

TABLE I  
SERUM PROTEINS IN RATS FED DIFFERENT AMOUNTS OF CASEIN OR OF WHEAT  
GLUTEN IN A PURIFIED DIET OVER A PERIOD OF FOUR WEEKS

Nitrogen intake (grams)	Serum proteins					
	Albumin (A)	Globulins (C)				A/C
		$\alpha_1$	$\alpha$	$\beta$	$\gamma$	
		(grams %)				
Casein						
0.99	1.87	0.89	0.10	1.20	0.60	0.57
1.62	2.22	0.71	0.57	1.14	0.85	0.68
4.60	2.43	0.70	0.61	1.21	0.60	0.75
9.16	2.89	0.78	0.50	1.03	0.60	0.95
10.41	2.62	0.77	0.60	1.27	0.53	0.82
Wheat gluten						
1.20	2.24	0.61	0.58	1.07	0.90	0.71
1.85	2.43	0.58	0.50	1.09	0.98	0.77
4.20	2.91	0.69	0.59	1.17	0.07	0.99
7.55	3.47	0.65	0.50	1.13	0.75	1.15

tively low albumin/globulin ratio in animals fed casein the ratios being higher in rats fed wheat gluten even though the growth of those fed the gluten was poor.

## C PROTEIN EFFICIENCY AND GROWTH OF NEOPLASMS

Three transplanted tumors in rats have been used to study the effect of diet upon growth of neoplasms (Allison *et al.* 1956). The Flexner-Jobling carcinoma transplanted into the rat for example responded to dietary protein in much the same way as a growth response of a normal tissue. Twenty eight days after transplantation this carcinoma had grown to 5.3 gm in rats fed wheat gluten. The tumor reached 11.3 gm in rats fed casein but was 2.0 gm in size in animals fed egg proteins. The plasma lipids in these animals were essentially normal. A transplanted sarcoma on the other hand grew to 24.7 gm in rats fed wheat

gluten, to 30.4 gm in those fed casein but was reduced to 20 gm in animals fed egg. Similarly, the Walker carcinoma 256 grew at maximum rate in rats fed 12% casein, being reduced in size in those fed either 12% wheat gluten or 12% egg. There was a marked increase in lipid migrating electrophoretically with the plasma globulins in rats with either the sarcoma or the Walker neoplasm. Both of these tumors developed most rapidly in rats fed 12-18% casein in the diet, the rate of development being reduced in animals fed smaller or larger amounts of casein. The difference in the rate of development with casein nitrogen intakes was correlated largely with the early establishment and growth of the tumor to reach the stage of most rapid growth, an early establishment that could be called the induction period. Further studies demonstrated that supplementing the casein with methionine increased the length of this induction time, thereby slowing down the development of the tumor. Supplementing with methionine plus guanidinacetic acid reduced the rate of development still more by not only increasing the induction time but also reducing the rate of growth of the tumor during the semi logarithmic phase (Allison *et al.*, 1956, 1957). These results of diet upon tumor development emphasize the importance of kinds and amount of dietary protein upon the dynamic state of the living system, a dynamic state where some tissues may at times grow at the expense of others.

#### D NITROGEN BALANCE AND GROWTH

Gain in body weight is not always a good measure of the growth of new tissue proteins. Some puppies, for example, were fed a purified diet containing either egg casein, or wheat gluten as the source of protein (Allison, 1949). The grams gain in body weight per gram of nitrogen eaten was approximately 9 regardless of the source of protein but the nitrogen balance per gram of nitrogen intake varied with the source, being 0.5 in dogs fed the egg protein, 0.43 in those fed casein, and 0.18 in those fed wheat gluten. The animals fed egg were lean and lively while those fed the wheat gluten were fat and less active. In general, the more positive the nitrogen balance produced by the dietary source, the better the pattern of amino acids for growth. However, correlation of amino acid patterns with nitrogen balance must take into consideration the concept of adaptation discussed previously in this report. A well balanced pattern with adequate intake of nitrogen should sustain growth and positive balance in young animals. Shifting from one pattern to another may produce a response into positive balance but if the pattern is not adequate for sustained protein anabolism the nitrogen balance will not be maintained but may even drift into negative

balance. The living system does have remarkable abilities to adapt to dietary situations but if the imbalances in the diet are too great whether they be between amino acids or between other constituents in the diet, the system develops a condition called malnutrition. Possibly the principle result of malnutrition is an inefficient living system inefficient in its responses to endogenous and exogenous changes in environment. Thus the maintenance of balance in all dietary constituents for the development of the most efficient living system is the function of good nutrition.

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## CHAPTER 6

# Dietary Proteins and Synthesis of Tissue Proteins

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## I INTRODUCTION

The body of an animal contains the following primary constituents: water, inorganic salts, lipids, proteins, and carbohydrates. Among these classes of compounds, proteins may be considered the most important because they perform a great number of physiological functions. For example, all pituitary hormones which control the secretion of most of the other important endocrine glands are proteins or polypeptides. Enzymes and antibodies are likewise proteins. During the life processes, these protein molecules are constantly destroyed and must be replaced by synthesis from amino acids derived from food. In order to utilize these amino acids, it is necessary that the so-called essential ones be available to the various tissues of the body. The works of Rose (1935), Madden and Whipple (1940), and Frazier *et al.* (1947) demonstrated that among numerous amino acids isolated from proteins, only 10 of them are essential to animals like dogs or rats for the promotion of growth, for the maintenance of nitrogen balance, and for the regeneration of plasma proteins. These amino acids are considered essential because the animals are not able to synthesize them in the body from the materials in the diet at a speed commensurate with the demands for normal growth. Such a definition is recognized by Borman *et al.*

(1946) may not include other important physiological functions such as reproduction, lactation, or detoxication. It follows that the requirement for an essential amino acid may vary according to the criterion employed to measure it and to the species of animals studied. For example, lysine is essential for the growth of young rats but according to Mitchell (1947) this amino acid is not needed for the maintenance of nitrogen equilibrium in normal, sexually mature rats. On the other hand, lysine is essential for the maintenance of nitrogen equilibrium in adult dogs (Anderson and Allison, 1947). In spite of such variations, a prime prerequisite for the nutritional adequacy of a dietary protein is the presence of sufficient amounts of all the essential amino acids.

The importance of availability of all the essential amino acids at the appropriate time and at the site of synthesis has been demonstrated in two types of experiments. In the first Elman (1939) showed that the intravenous injection of tryptophan to dogs previously fed an acid hydrolyzate of casein deficient only in this amino acid can bring about nitrogen retention which otherwise will not take place. However the supply of tryptophan must not be unduly delayed. In the second Harte *et al* (1948) and Geiger (1948) fed animals two diets each containing a deficient protein, which could adequately supplement one another for growth requirements. The animals received no benefit when the second diet was given long after the first had been ingested. However if the diets were offered simultaneously the animals could utilize such a mixture for the synthesis of body tissues. Thus the nutritive value of an intact protein depends not only on the presence of the essential amino acids but also on the relative rates at which they are released by the digestive enzymes. Therefore, it has been suggested by Melnick *et al* (1946) that the increase of biological value of soybean proteins after heating is due to the greater ease of liberating the limiting amino acid methionine.

From the important contributions of these investigations it can be concluded that the presence of all the essential amino acids in a dietary protein is a prerequisite for adequate nutrition. On the other hand, adequate supply of all the essential amino acids at the proper site in the body and at the appropriate time is all that is required. The deficiency of poor proteins low in the content of certain essential amino acids can be made up by an increase of dietary intake or by supplementation of the deficient amino acids. However, there is evidence which indicates that this is not in accordance with experimental data. Studies of Woolley (1947) and Womack and Rose (1946) suggest that in addition to the essential amino acids some unknown nutritional factor is needed for optimal growth. The growth experiments necessary to

substrate such a hypothesis is complicated by the synthesis of yet unidentified vitamins by the bacterial organisms in the intestinal flora.

In this communication data will be presented to demonstrate that the synthesis of tissue proteins depends not only on the presence of the essential amino acids in adequate amounts but also on the presence of certain accessory factors in the diet.

## II THE EFFECT OF DIETARY PROTEINS ON THE SYNTHESIS OF PLASMA PROTEINS

### A DEPLETION OF PLASMA PROTEINS

Chow *et al* (1945) studied the effects of protein depletion on the plasma proteins of dogs. Protein depletion was achieved by offering a group of well fed normal dogs a protein free diet containing sufficient calories and vitamins to meet the daily requirements. Data shown in Table I (Group B) demonstrate that following 6 to 8 weeks of feeding this diet the plasma protein concentration of the animals dropped from 6.8 to 5.0 gm per 100 ml. Since the plasma volume likewise decreased about 20% the decrement in total circulating plasma proteins was greater than would be calculated on the basis of changes solely in plasma protein concentration. Electrophoretic analysis of plasma of dogs made before and after depletion demonstrated that the total circulating albumin suffered a marked loss as compared to the total circulating globulins. It is interesting to note that although the total circulating globulins decreased during protein free feeding the total circulating  $\alpha$  globulins tended to increase. The maintenance of the total circulating  $\alpha$  globulins in the depleted state even though the other plasma protein components have been reduced is of interest although its physiological role is still obscure. Also included in Table I are the results of the electrophoretic analysis of the plasma of dogs protein depleted by two other methods. The first method was to restrict the caloric intake by offering the animals a nutritionally adequate diet at the level of 30 rather than 80 calories per kilogram body weight per day (Group C). In the second method protein depletion was achieved by daily plasmapheresis while the animals were fed a protein free diet (Group A). It may be seen that the qualitative and quantitative changes in the various plasma proteins are essentially the same although the time necessary to bring about this result varied according to the method of depletion. Under such experimental conditions the decrease in total circulating albumin reached a plateau of approximately 30% of the original values and the albumin/globulin ratio decreased to 0.27.

Before discussing the possible effects of dietary proteins on the rate

TABLE I  
COMPOSITION OF PLASMA PROTEIN OF DOGS BEFORE AND AFTER PROTEIN DEPLETION BY DIFFERENT METHODS

Method of depletions <sup>a</sup>	Number of dogs	Treatment	Time of treatment (days)	Plasma volume (ml)	Plasma protein (gm %)	Total proteins (gm)	Total circulating plasma proteins					A/C
							Albumin (gm)	Globu- lins (gm)	$\alpha_1 + \alpha_2$ Globu- lins (gm)	$\gamma$ Globu- lins (gm)		
A	15	Control		473	6.21	29.4	12.4	16.5	1.5	2.5	0.75	
	15	Depleted	10	427	4.48	19.2	4.0	14.7	1.9	1.8	0.27	
B	15	Control		603	6.78	40.6	16.0	24.9	6.6	3.0	0.67	
	15	Depleted	42-53	510	5.04	25.6	5.3	19.4	6.8	3.0	0.27	
C	5	Control		500	6.37	31.9	12.7	19.0	4.8	2.9	0.67	
	5	Depleted	14-21	425	4.52	19.2	3.8	14.1	4.5	1.6	0.27	

<sup>a</sup> A = Plasmapheresis and protein free feeding B = Protein free feeding and C = Feeding a diet low in calories

of repletion it is appropriate to consider the importance of the degree of depletion of protein reserves and of the mode of administration of the dietary proteins. The albumin/globulin ratio of 0.27 which was observed in the protein depleted dogs could be further lowered only by prolonged protein deprivation. It is interesting to note that only at this time was a decrease in the total circulating  $\alpha$  globulins observed. Feeding casein or lactalbumin or the hydrolyzates of either protein at this stage failed to bring the animals into nitrogen equilibrium regardless of the quantity of intake (within 0.30 to 1.0 gm nitrogen per kilogram body weight per day) unless 15 units of liver extract were given simultaneously for at least 5 days. The results of a typical experiment in which animals were fed a casein diet are shown in Table II. These

TABLE II  
THE EFFECT OF INTRAMUSCULAR INJECTION OF LIVER EXTRACT ON THE NITROGEN RETENTION BY DOGS SEVERELY DEPLETED OF PROTEIN RESERVES<sup>a</sup>

Group	No of dogs	Weeks on protein free diet	Albumin/globulin ratio	Days of feeding	Nitrogen <sup>b</sup> intake	Nitrogen <sup>b</sup> output	Nitrogen <sup>b</sup> balance
A	3	18	0.12	10	300	380	-80
B	3	20	0.14	10	1000	1100	-100
C	3	18	0.14	10	300	160	+140

<sup>a</sup> All animals were given 60 cal per kilogram body weight per day. One milliliter of 15 units of liver extract was given daily for first 5 days to animals in Group C.

<sup>b</sup> These figures are averages expressed as milligrams per kilogram body weight per day.

data demonstrate that increasing the nitrogen intake from 300 to 1000 mg per kilogram body weight per day failed to bring about nitrogen retention. Only the administration of liver extract would return these severely protein depleted animals to an anabolic state. Alper *et al* (1950) studied the utilization of an enzymatic digest of casein by dogs depleted of protein either by prolonged protein free feeding alone or in conjunction with plasmapheresis. During the first part of the experiment the pyrogen free hydrolyzate containing 40% of its total nitrogen as amino nitrogen was administered parenterally for a prolonged period (4 weeks) at a level of high caloric intake (supplied orally in the form of protein free diet at 70-90 calories per kilogram body weight per day) to insure a surplus amount of nutrients. Subsequently the same animals received the same hydrolyzate (dried by lyophilization) orally for an additional period. Nitrogen retention and plasma protein regeneration were determined during both phases of the experiment.

These results (Table III) demonstrate two interesting points (1)

Dogs fed a protein free diet (Group 1) were in more positive nitrogen balance than those depleted by plasmapheresis (Group 2), when the hydrolyzate was given intravenously. During this period, both groups regenerated plasma proteins. (2) All dogs retained more nitrogen when the casein diet was fed *per os*.

TABLE III

THE EFFECT OF ROUTE OF ADMINISTRATION OF CASEIN HYDROLYZATE ON NITROGEN BALANCE AND PLASMA PROTEIN REGENERATION OF DOGS

Group	No. of dogs	Period I (4 weeks of iv feeding)		Period II (4 weeks of oral feeding)	
		Nitrogen balance <sup>b</sup>	Gain in plasma protein <sup>c</sup>	Nitrogen balance <sup>b</sup>	Gain in plasma protein <sup>c</sup>
1	6	+0.071 ± 0.023	6.6 gm	+0.165 ± 0.027	6.3 gm
2	4	+0.023 ± 0.018	1.8 gm	+0.145 ± 0.015	3.5 gm

<sup>a</sup> Group 1 = dogs depleted of proteins by protein free feeding for 6-8 weeks.  
Group 2 = dogs depleted of proteins by restricted plasmapheresis.

<sup>b</sup> Average nitrogen balance expressed as grams of nitrogen per kilogram body weight per day with standard error.

<sup>c</sup> Gain in total circulating plasma proteins in grams. The gain during the second period represents the additional increase of total circulating plasma proteins following the oral administration.

The authors concluded:

(1) In hypoproteinemia effected by restricted plasmapheresis, the 'reserve protein stores' of the experimental animals were not permanently depleted although plasma protein concentration and volume were markedly reduced. On the other hand, the reserve protein stores of the animals fed a protein free diet over a long period were reduced first and then the plasma proteins depleted. Thus, animals depleted by the latter method might be expected to show a different utilization of the parenterally administered nitrogen.

(2) The higher retention of orally administered nitrogen following partial repletion by parenterally administered nitrogen indicates the importance of the pattern of amino acids and peptides available to the animal for the synthesis of its body tissues.

However, it should be pointed out that renal loss of amino acids and polypeptides may explain the lower retention of parenterally administered nitrogen in these dogs.

#### B. REPLETION WITH DIFFERENT DIETARY PROTEINS

The studies of Madden *et al* (1938), Melnick *et al* (1936), Melnick and Cowgill (1937), Weech and Goettsch (1938, 1939), Weech (1942),

and Cox and Mueller (1944) suggested that various food proteins differ qualitatively in their ability to promote synthesis of plasma proteins. For example, by means of the plasmapheresis technique Madden *et al* (1938) demonstrated that some dietary proteins may favor the production of plasma albumin and others may favor the production of plasma globulins. The limitations of these studies were the inherent inadequacy of the chemical methods for the determination of albumin and globulins and the lack of information of plasma volumes in some reports. With the advance of the electrophoretic analyses for the determination of plasma protein components and with the improvement in the accuracy and reliability in the determination of plasma volume Chow *et al* (1949) re-examined the question of whether various common dietary proteins (such as egg white, whole egg, lactalbumin, casein, and wheat gluten) of different nutritive values as measured by growth or nitrogen balance tests differ in their ability to regenerate plasma proteins. The protein-depleted dog is a very valuable experimental asset because of its availability and the reproducibility of results thus obtained. To this end Chow *et al* (1949a) fed to several groups of protein-depleted animals diets containing 1 of the 5 test proteins so that each animal received 0.35 gm of nitrogen and 80 calories per kilogram body weight per day for a period of at least 4 weeks. The exception was that 0.6 gm of wheat gluten nitrogen was given instead of 0.35 gm because of its low biological value. Determinations of total circulating proteins as well as albumin and globulins were made before feeding and again 2 and 4 weeks after feeding. The results of this experiment are shown in Table IV.

### 1 Repletion of Total Circulating Plasma Proteins

These data demonstrate that supplementation of the protein-free diet with any one of the 5 proteins or 2 hydrolyzates stimulated an increase in the total circulating plasma proteins. The difference in the per cent increases was not very marked among the test substances even though the nutritive values as measured in terms of growth efficiency differed by several fold. Furthermore, there appeared to be a lack of correlation between nitrogen balance index and plasma protein regeneration properties. For example, lactalbumin with a nitrogen balance index of unity did not stimulate as effectively the synthesis of plasma proteins as did casein or whole egg proteins with indexes of 0.80 and 0.95 respectively. Similarly, egg white which has the highest biological value among the 5 proteins was inferior in bringing about regeneration of plasma proteins following 2 weeks of repletion. A comparison of the whole proteins and their respective hydrolyzates showed that the plasma protein regeneration properties of casein were not improved by tryptic digestion while



TABLE IV

EFFECTS OF ORAL FEEDING OF VARIOUS PROTEINS ON TOTAL ALBUMIN AND GLOBULINS OF DOGS DEPLETED IN PROTEINS<sup>a</sup>

Protein fed	No dogs used	Total circulating protein <sup>b</sup>				Total circulating albumin <sup>b</sup>				Total circulating globulins <sup>b</sup>				A/G	
		after feeding for		after feeding for		after feeding for		after feeding for		after feeding for		after feeding for		after feeding for	
		2 wk	4 wk	2 wk	4 wk	2 wk	4 wk	2 wk	4 wk	2 wk	4 wk	0 wk	2 wk	4 wk	4 wk
Egg white	10	117 ± 5	132 ± 17	117 ± 8	133 ± 12	118 ± 5	126 ± 10	118 ± 5	126 ± 10	118 ± 5	126 ± 10	0.28	0.27	0.26	0.26
Lactalbumin	5	115 ± 10	128 ± 10	185 ± 21	252 ± 23	100 ± 9	100 ± 9	100 ± 9	100 ± 9	100 ± 9	100 ± 9	0.22	0.42	0.58	0.58
Whole egg	5	148 ± 7	154 ± 7	206 ± 21	289 ± 22	138 ± 6	129 ± 7	138 ± 6	129 ± 7	138 ± 6	129 ± 7	0.19	0.28	0.43	0.43
Casein	4	138 ± 9	111 ± 5	177 ± 17	216 ± 19	130 ± 8	124 ± 4	130 ± 8	124 ± 4	130 ± 8	124 ± 4	0.25	0.33	0.39	0.39
Wheat gluten	5	135 ± 4	135 ± 4	162 ± 6	168 ± 7	130 ± 5	129 ± 5	130 ± 5	129 ± 5	130 ± 5	129 ± 5	0.17	0.21	0.22	0.22
Lactalbumin hydrolyzate	6	141 ± 8	145 ± 6	230 ± 5	362 ± 8	115 ± 6	98 ± 7	115 ± 6	98 ± 7	115 ± 6	98 ± 7	0.21	0.44	0.85	0.85
Casein hydrolyzate	5	138 ± 7	150 ± 3	223 ± 4	238 ± 8	119 ± 11	194 ± 10	119 ± 11	194 ± 10	119 ± 11	194 ± 10	0.22	0.41	0.51	0.51

<sup>a</sup> All the proteins were given at a level of 0.35 gm N per kilogram body weight per day except wheat gluten which was given at a 0.6 gm level<sup>b</sup> The total circulating proteins before feeding are taken as 100% The figures after the ± signs are standard errors

the tryptic digest of lactalbumin was definitely superior to the whole protein. This might be explained by the fact that casein is rapidly hydrolyzed by the proteolytic enzymes normally present in the digestive tracts of the animals whereas lactalbumin is not.

## 2 Repletion of Total Circulating Albumin

The 5 test proteins varied in their ability to stimulate the production of albumin. For example, the total circulating albumin of dogs fed either egg white or whole egg during the first 2 weeks of the repletion period was increased 117% and 206% respectively. The regeneration rates of albumin by the hydrolyzates were significantly greater than those of the corresponding proteins (185% against 230% for lactalbumin and its hydrolyzate, 177% against 223% for casein and its hydrolyzates respectively).

## 3 Repletion of Total Circulating Globulins

A careful examination of data on the globulin regeneration shows that while 4 of the proteins administered stimulated the production of globulins in varying degrees, dogs fed lactalbumin or its hydrolyzate failed to gain in globulins. This difference contributes to the high albumin/globulin ratio seen in the animals fed lactalbumin or its hydrolyzate during the period of repletion. However, the data indicate that the high albumin/globulin ratio is also a reflection of a greater rate of regeneration of the total circulating albumin in these animals.

These differences which may be attributed to the ingestion of casein hydrolyzate and lactalbumin hydrolyzate during repletion are shown clearly in Table V. Repletion with casein hydrolyzate or lactalbumin hydrolyzate resulted in a return of the total circulating albumin to essentially the control values. However, it may be noted that the plasma albumin of the animals fed casein hydrolyzate was slightly lower than the initial levels, although slightly higher values were observed in the dogs fed lactalbumin hydrolyzate. There was little change in the total circulating  $\alpha$  globulins during depletion or repletion. The most significant difference attributed to feeding these diets was the increase above the initial values in the "other globulins" observed in the dogs repleted with casein hydrolyzate. It may be seen that this difference was reflected in the albumin/globulin ratios.

The first attempt to explain these observations was based on the possible difference in the amino acid patterns of the casein and lactalbumin hydrolyzates and perhaps a similarity of amino acid contents of these protein preparations and dog plasma proteins. Therefore, analyses for the essential amino acids of the 2 hydrolyzates as well as of the

electrophoretically homogeneous albumin and of the total globulins of dog plasma were performed. These results (Bolling *et al.*, 1947) demonstrated that the 2 hydrolyzates differed most significantly in their ratios of cystine to methionine. Since less than 5% of the nitrogen

TABLE V

THE AVERAGE TOTAL CIRCULATING ALBUMIN  $\alpha$   $\beta$  AND  $\gamma$  GLOBULINS AND ALBUMIN/GLOBULIN RATIOS IN THE CONTROL DEPLETED AND REPLETED CONDITION ON 5 DOGS REPLETED WITH A CASEIN HYDROLYZATE AND 6 DOGS REPLETED WITH A LACTALBUMIN HYDROLYZATE\*

Condition	Circulating albumin gm / m <sup>2</sup>	Circulating globulin			A/G
		Alpha gm / m <sup>2</sup>	Gamma gm / m <sup>2</sup>	Other gm / m <sup>2</sup>	
Casein hydrolyzate					
Control	25.7	9.7	4.8	19.6	0.75
Depleted	7.6	10.1	3.9	20.2	0.22
Repleted	23.4	10.9	6.0	28.7	0.51
Lactalbumin hydrolyzate					
Control	28.4	8.1	6.0	21.5	0.80
Depleted	6.9	8.0	3.8	21.4	0.21
Repleted	29.5	7.3	5.3	22.3	0.85

\* Each dog received 0.35 gm of hydrolyzate nitrogen per kilogram of body weight per day for 30 days. The average total circulating albumin and globulins are expressed as gm/m<sup>2</sup> of body surface.

retained was utilized for the synthesis of plasma proteins (Table VI), it is unlikely that the inability of dogs fed lactalbumin hydrolyzate to induce the production of globulins was due to any limiting amino acids fed. Furthermore, the analyses of albumin and globulins did not reveal any specific requirements of certain amino acids which the hydrolyzates had to supply for the synthesis of the 2 fractions of plasma proteins.

The inferiority of casein as compared to lactalbumin in promoting the growth of young rats (Supplee and Clark, 1946), or in supporting nitrogen balance in dogs (Allison, 1946), can be compensated for by feeding animals a higher level of casein. It was, therefore, of interest to ascertain whether the differences in the production of specific protein components in plasma by these 2 milk proteins is similarly dependent on the amount of absorbed nitrogen. Studies were undertaken which involved feeding a group of protein depleted dogs casein hydrolyzate at a nitrogen level sufficient to stimulate a rapid and pronounced regeneration of plasma proteins. For comparison three other groups of protein depleted animals were fed three different levels of a tryptic digest of lactalbumin, so that the returned nitrogen was either greater,

TABLE VI  
THE LEVELS OF ABSORBED NITROGEN AND THE PLASMA PROTEIN REGENERATION

Protein supplement	No dogs used	Level of intake <sup>a</sup>	Nitrogen balance <sup>b</sup>	Retained biological value <sup>c</sup> (× 100)	Plasma protein nitrogen <sup>d</sup> %	Total circulating globulins <sup>e</sup>	
						3 wk	6 wk
Lactalbumin hydrolyzate	5	0.35	+0.186	0.80	1.6	100 ± 9.0	100 ± 9.3
Lactalbumin hydrolyzate	4	0.25	+0.118	0.80	3.0	106 ± 5.2	105 ± 6.5
Lactalbumin hydrolyzate	4	0.15	+0.072	1.00	2.7	97 ± 9.6	105 ± 7.0
Casein hydrolyzate	5	0.35	+0.117	0.50	1.5	111 ± 5.7	120 ± 3.0
Casein hydrolyzate + 1% methionine	3	0.35	+0.175	0.70	1.5	116 ± 1.8	118 ± 1.0
Casein hydrolyzate + 2% methionine	3	0.35	+0.165	0.70	5.0	113 ± 10.6	136 ± 9.1

<sup>a</sup> Level of intake is expressed as grams nitrogen per kilogram body weight per day

<sup>b</sup> Nitrogen balance is the difference between nitrogen intake and nitrogen excretion expressed in grams nitrogen per kilogram body weight per day

<sup>c</sup> Biological value. <sup>†</sup> calculated according to the equation of Allison (1946) NB = (BV) (AN) -- (EN) (See text)

<sup>d</sup> Per cent of the total absorbed nitrogen used for the synthesis of total plasma protein

<sup>e</sup> Calculated as per cent of the amount determined prior to protein feeding, with standard error

approximately equal to, or less than that retained by dogs receiving the casein hydrolyzate. In addition, two other groups of dogs were fed the same level of casein hydrolyzate as the first group, but were given 1 or 2% methionine as supplement in order to increase the nitrogen retention to approximately that of the animals receiving the highest level of lactalbumin hydrolyzate. The regeneration of various plasma protein components and the nitrogen retention were measured throughout the feeding period.

All six diets were adequate in bringing about nitrogen retention in the protein depleted dog (Table VI). At the level of nitrogen intake 0.35 gm per kilogram body weight per day at least 80% of the lactalbumin hydrolyzate nitrogen was retained as compared to 50% of the casein hydrolyzate nitrogen. The addition of 1 or 2% methionine to the casein diet increased the retention to only 70%. Furthermore, dogs fed any one of the three levels of lactalbumin hydrolyzate regenerated no globulins, whereas those fed casein hydrolyzate produced globulins irrespective of supplementation of methionine.

The data presented above led to the belief that the differences in the plasma protein regeneration properties of casein and lactalbumin were neither due to the amino acid composition nor to the amount of amino acids retained by the animals. Therefore, some nutritional factors other than amino acids may play a role in directing the synthesis of plasma proteins. To investigate this possibility, a casein hydrolyzate was fractionated with alcohol, and the addition of 1% of the fraction, soluble in 60% but insoluble in 80% alcohol to a lactalbumin hydrolyzate improved the globulin regeneration properties (see Table VII). It is unlikely that supplementation to the extent of 1% was enough to sufficiently alter the amino acid pattern of the lactalbumin hydrolyzate.

TABLE VII

THE EFFECT OF ADDITION OF A FRACTION OF CASEIN HYDROLYZATE TO LACTALBUMIN HYDROLYZATE ON PLASMA PROTEIN REGENERATION

Level of feeding	No of dogs	TCA <sup>a</sup>	TCG <sup>b</sup>
Lactalbumin hydrolyzate 0.35 gm N/kg	5	196 ± 11	100 ± 4
Lactalbumin hydrolyzate + 1% CHA <sup>c</sup> 0.35 gm N/kg	5	206 ± 8	120 ± 6
Casein hydrolyzate 0.35 gm N/kg	5	210 ± 12	127 ± 8

<sup>a</sup> TCA = total circulating albumin in % of the amount determined prior to protein feeding with standard error after 4 weeks of feeding.

<sup>b</sup> TCG = total circulating globulins in % of the amount determined prior to protein feeding with standard error after 4 weeks of feeding.

<sup>c</sup> CHA = the fraction of casein hydrolyzate soluble in 60% alcohol but insoluble in 80% alcohol.

TABLE VIII  
SOME CHEMICAL AND BIOLOGICAL CHARACTERISTICS OF THE CASFIN HYDROLYZATE FRACTION SOLUBLE IN 60% ALCOHOL  
BUT INSOLUBLE IN 80% ALCOHOL

Analyses	Results	Method	Author
Total N as % of dry weight	14.6	Micro-kjeldahl	—
Amino nitrogen as % of total nitrogen	68	Ninhydrin reaction	Dillon
Microbiological assays			
(a) for LLD	traces	LLD	Shorb
(b) for streptogenin	none	<i>L. casei</i>	Sprince and Woolley

These data therefore, indicate the existence of a fraction of casein hydrolyzate which, when added to lactalbumin hydrolyzate in a small quantity, was effective in globulin regeneration

Some of the chemical and biological characteristics of this fraction were also determined (see Table VIII). Chemical analyses show that it contained 14.6% total nitrogen by weight. Sixty eight per cent of the total nitrogen was amino nitrogen according to the ninhydrin method. Microbiological assay for Wolley's streptogenin factor using *L. casei* gave negative results. This is interesting since the original casein hydrolyzate contained 5 units per gram and the lactalbumin hydrolyzate contained about 1 unit per gram. Microbiological assay for crystalline vitamin B<sub>12</sub> like activity using *Lactobacillus lactis* Dorner likewise showed the presence of only a minute amount of this factor (less than 10 µg per gram of this fraction). Nevertheless, supplementation of a soybean diet with this fraction to the extent of 1 part per 100,000 stimulated the growth rate (Table IX) of weanling rats raised by mothers

TABLE IX

THE EFFECT OF THE FRACTION OBTAINABLE FROM CASEIN HYDROLYZATE ON THE GROWTH RATE OF YOUNG RATS

Diets <sup>a</sup>	Supplement	Amount added mg /100 gm ration	Increase in body weight over control (in grams)		
			1 wk	2 wk	3 wk
Soybean	Casein factor	0.5	5	12	18
Soybean	Casein factor	1.0	8	14	22
Soybean	Casein factor	2.0	8	15	21
Soybean	{ Casein factor	2.0	14	23	38
	{ Crystalline B <sub>12</sub>	1.0 (µg)			

<sup>a</sup> Soybean diet 60% soybean meal 30% sucrose 4% salt mixture 6% Mazola

fed a soybean diet. The addition of crystalline vitamin B<sub>12</sub> at a level to produce a maximum response accentuated the growth rate even further. This fact may be considered additional evidence that this factor is distinct from vitamin B<sub>12</sub>.

### III THE EFFECT OF DIETARY PROTEINS ON THE CONCENTRATION OF PLASMA CHOLINESTERASE OF RATS

#### A GROWING ANIMALS

Since all enzymes thus far isolated are proteins it seems reasonable to expect that the synthesis of enzymes such as plasma cholinesterase, could be effected by the ingestion of dietary proteins. To test this hypothesis, weanling rats were fed diets containing various dietary pro

tein preparations for several months (Burrows 1958). In general the diets contained 20% protein (Kjeldahl nitrogen  $\times 6.25$ ), 12% Mazola oil 4% salts 1% sucrose to make up 100% and adequate amounts of the known vitamins. In experiments in which the protein contents of the diets were increased sucrose was withdrawn in amounts equal to the weight of the added protein preparation. The animals were fed the various test diets and offered water *ad libitum*. All animals were weighed weekly for the first 2 months of the experimental period and bimonthly thereafter. Following the feeding period (150 days) all animals were bled by cardiac puncture under light ether anesthesia. The cholinesterase activity in 0.3 ml of plasma was determined by the method of Ammon (1930) as modified by Mazur and Bodansky (1946). In order to make the cholinesterase activity of samples determined on different days comparable all determinations were referred to a pooled normal horse serum kept frozen in aliquots and used as the standard for each assay. The results of such a study are presented in Table 1.

TABLE 1  
THE EFFECT OF DIETARY PROTEINS ON THE PLASMA CHOLINESTERASE OF RATS

Dietary protein preparations	Plasma cholinesterase (RP units/0.3 ml)	
	Females	Males
Wheat gluten	0.60 $\pm$ 0.4	0.56 $\pm$ 0.4
Wheat gluten + supplement <sup>b</sup>	1.30 $\pm$ 10	—
Soybean meal	1.18 $\pm$ 11	—
Casein	1.31 $\pm$ 07	0.70 $\pm$ 0.4
Stock diet	1.36 $\pm$ 13	—
Egg albumin <sup>c</sup>	1.40 $\pm$ 10	—
Whole desiccated liver	1.42 $\pm$ 15	—
Lactalbumin	1.59 $\pm$ 11	0.67 $\pm$ 0.5
Beef muscle	1.68 $\pm$ 07	0.62 $\pm$ 0.3

<sup>a</sup> All diets contained 20% protein (Kjeldahl N  $\times 6.25$ )

<sup>b</sup> Supplement contained 17.6 gm / lysine HCl H<sub>2</sub>O and 3.5 gm / methionine per kilogram diet

Supplemented with 1 mg biotin

There were no marked differences in the plasma cholinesterase activity of male rats although the growth rates of the animals differed strikingly. On the other hand the ingestion of various dietary proteins resulted in marked differences in the cholinesterase levels in the plasma of female rats. In general these levels were related to the quality of the protein fed. For example weanling female rats fed a poor protein *viz.* wheat gluten had both inferior growth rates and plasma cholinesterase activity. The addition of lysine and methionine to the diet improved the growth



rate and at the same time elevated the level of the enzyme. Furthermore, the plasma cholinesterase activities of female rats fed diets containing proteins which support adequate growth do not differ significantly from those fed the diet containing casein. The outstanding exception to this is the high level of cholinesterase activity in the plasma of female rats fed the beef muscle diet. Thus, even though the ages, body weights, and growth rates were indistinguishable, the plasma cholinesterase activity was higher in the female animals fed the beef muscle as compared with that of those fed the casein diet. It is interesting that in spite of an observed impairment of growth the enzyme activity of the female rats fed the whole desiccated liver diet was essentially equal to that of animals fed the casein diet. The higher plasma cholinesterase activity of the animals fed the beef muscle as compared to casein diet cannot be explained on the basis of hemoconcentration since the concentration of plasma proteins of the two groups was  $6.05 \pm 0.16$  gm per 100 ml and  $5.80 \pm 0.20$  gm per 100 ml respectively. Furthermore, the cholinesterase activities of mixtures of plasma of rats fed the casein and beef muscle diets were determined. The experimentally determined enzymatic activities were equal within experimental error to the sum of the activities of each plasma sample. Thus, no evidence is available to indicate that the observed differences in the plasma cholinesterase may be attributed to differences in the concentration of activators or inhibitors. Finally, since the differences attributed to diet were equally as great when the enzyme activity was determined by a colorimetric rather than the manometric method, these results are not due to inherent errors in the methods of analysis. Therefore, it seems likely that the increased plasma cholinesterase activity observed in female rats fed the beef muscle diet represents an increased amount of the enzyme per se.

The lack of a demonstrable difference in the enzymatic activity of male rats may be explained on the basis of the existence of two forms of cholinesterase, namely, true cholinesterase and pseudocholinesterase. The relative amounts of these enzymes have been found to be dependent upon sex and the greater amount of plasma cholinesterase activity in the mature females as compared to mature males has been attributed to the higher level of pseudocholinesterase (Everett and Sawyer, 1947). The method used in this study determines primarily pseudocholinesterase although true cholinesterase is not completely inhibited. Therefore, the lack of a sex difference in the plasma cholinesterase of animals fed wheat gluten (Table X) indicates an unusually low level of pseudocholinesterase in these female rats. In addition the effect of the ingestion of various dietary protein preparations on plasma cholinesterase was

not evident in male animals. Taken as a whole these results suggest that the amount of plasma pseudocholinesterase rather than true cholinesterase is increased by ingestion of certain dietary protein preparations.

The preceding experiment indicated that some unknown nutritive factors may be present in beef muscle and perhaps whole desiccated liver, but absent in the other protein preparations tested. If this were true then the addition of beef muscle or whole desiccated liver to a diet containing adequate amounts of the essential amino acids but devoid of these nutrients should result in an increased level of plasma cholinesterase. On the other hand the supplementation of such a diet with egg albumin or casein should be ineffective. In order to test this hypothesis the 20% casein diet used previously served as the basal diet to which beef muscle, whole desiccated liver, egg albumin or additional casein was added. The results of this experiment (Table XI) demon-

TABLE XI  
THE EFFECT OF SUPPLEMENTING A CASEIN DIET WITH DIETARY PROTEINS ON THE PLASMA CHOLINESTERASE OF FEMALE RATS

Diets	% Protein <sup>a</sup>	Gain in weight (gm) <sup>b</sup>	Plasma cholinesterase (RP units/0.3 ml.)
24% Casein	20	189 ± 8.0	1.32 ± .10
38% Casein	32	181 ± 6.1	1.17 ± .09
24% Casein + 15% egg albumin <sup>c</sup>	32	186 ± 6.2	1.27 ± .05
24% Casein + 3% beef muscle	23	180 ± 6.0	1.31 ± .09
24% Casein + 13% beef muscle	32	188 ± 7.0	1.58 ± .07
24% Casein + 4% whole desiccated liver	23	194 ± 6.9	1.60 ± .09
17% Whole desiccated liver + 24% casein	32	208 ± 4.0	1.95 ± .14
21% Beef muscle	20	174 ± 6.3	1.74 ± .09

<sup>a</sup> Protein content based on Kjeldahl N × 6.25

<sup>b</sup> After 150 days of feeding

<sup>c</sup> Supplemented with 1 mg. of biotin

strate that the gain in body weight of the animals fed the various diets for 150 days was essentially the same regardless of the protein supplement or the per cent of protein in the diet. However marked differences in the cholinesterase concentration in different groups were observed. For example supplementation of the basal casein diet with beef muscle or whole desiccated liver elevated the level of the enzymatic activity. Although feeding a diet supplemented with 4% whole desiccated liver

rate and at the same time elevated the level of the enzyme. Furthermore, the plasma cholinesterase activities of female rats fed diets containing proteins which support adequate growth do not differ significantly from those fed the diet containing casein. The outstanding exception to this is the high level of cholinesterase activity in the plasma of female rats fed the beef muscle diet. Thus, even though the ages, body weights and growth rates were indistinguishable, the plasma cholinesterase activity was higher in the female animals fed the beef muscle as compared with that of those fed the casein diet. It is interesting that in spite of an observed impairment of growth the enzyme activity of the female rats fed the whole desiccated liver diet was essentially equal to that of animals fed the casein diet. The higher plasma cholinesterase activity of the animals fed the beef muscle as compared to casein diet cannot be explained on the basis of hemoconcentration, since the concentration of plasma proteins of the two groups was  $6.05 \pm 0.16$  gm per 100 ml and  $5.80 \pm 0.20$  gm per 100 ml respectively. Furthermore, the cholinesterase activities of mixtures of plasma of rats fed the casein and beef muscle diets were determined. The experimentally determined enzymatic activities were equal within experimental error to the sum of the activities of each plasma sample. Thus, no evidence is available to indicate that the observed differences in the plasma cholinesterase may be attributed to differences in the concentration of activators or inhibitors. Finally, since the differences attributed to diet were equally as great when the enzyme activity was determined by a colorimetric rather than the manometric method, these results are not due to inherent errors in the methods of analysis. Therefore, it seems likely that the increased plasma cholinesterase activity observed in female rats fed the beef muscle diet represents an increased amount of the enzyme per se.

The lack of a demonstrable difference in the enzymatic activity of male rats may be explained on the basis of the existence of two forms of cholinesterase, namely true cholinesterase and pseudocholinesterase. The relative amounts of these enzymes have been found to be dependent upon sex and the greater amount of plasma cholinesterase activity in the mature females as compared to mature males has been attributed to the higher level of pseudocholinesterase (Everett and Sawyer, 1947). The method used in this study determines primarily pseudocholinesterase although true cholinesterase is not completely inhibited. Therefore the lack of a sex difference in the plasma Cholinesterase of animals fed wheat gluten (Table X) indicates an unusually low level of pseudocholinesterase in these female rats. In addition the effect of the ingestion of various dietary protein preparations on plasma cholinesterase was

not evident in male animals. Taken as a whole these results suggest that the amount of plasma pseudocholinesterase rather than true cholinesterase is increased by ingestion of certain dietary protein preparations.

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Diets	% Protein <sup>a</sup>	Gain in weight (gm.) <sup>b</sup>	Plasma cholinesterase (RP units/0.3 ml.)
21% Casein	20	189 ± 8.6	1.32 ± .10
38% Casein	32	181 ± 6.1	1.17 ± .09
24% Casein + 15% egg albumin	32	180 ± 6.2	1.27 ± .05
24% Casein + 3% beef muscle	23	180 ± 6.0	1.31 ± .09
24% Casein + 13% beef muscle	32	188 ± 7.0	1.58 ± .07
24% Casein + 4% whole desiccated liver	23	194 ± 6.9	1.60 ± .09
17% Whole desiccated liver + 24% casein	32	208 ± 4.0	1.95 ± .14
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<sup>a</sup> Protein content based on Kjeldahl N × 6.25

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strate that the gain in body weight of the animals fed the various diets for 150 days was essentially the same regardless of the protein supplement or the per cent of protein in the diet. However marked differences in the cholinesterase concentration in different groups were observed. For example supplementation of the basal casein diet with beef muscle or whole desiccated liver elevated the level of the enzymatic activity. Although feeding a diet supplemented with 4% whole desiccated liver

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not evident in male animals. Taken as a whole these results suggest that the amount of plasma pseudocholinesterase rather than true cholinesterase is increased by ingestion of certain dietary protein preparations.

The preceding experiment indicated that some unknown nutritive factors may be present in beef muscle and perhaps whole desiccated liver but absent in the other protein preparations tested. If this were true then the addition of beef muscle or whole desiccated liver to a diet containing adequate amounts of the essential amino acids but devoid of these nutrients should result in an increased level of plasma cholinesterase. On the other hand the supplementation of such a diet with egg albumin or casein should be ineffective. In order to test this hypothesis the 20% casein diet used previously served as the basal diet to which beef muscle whole desiccated liver egg albumin or additional casein was added. The results of this experiment (Table VI) demon-

TABLE VI  
THE EFFECT OF SUPPLEMENTING A CASEIN DIET WITH DIETARY PROTEINS ON THE PLASMA CHOLINESTERASE OF FEMALE RATS

Diets	% Protein <sup>a</sup>	Gain in weight (gm) <sup>b</sup>	Plasma cholinesterase (RP units/0.3 ml.)
24% Casein	20	189 ± 8.6	1.32 ± .10
38% Casein	32	181 ± 6.1	1.17 ± .09
21% Casein + 15% egg albumin <sup>c</sup>	32	186 ± 6.2	1.27 ± .05
24% Casein + 3% beef muscle	23	180 ± 6.0	1.31 ± .09
24% Casein + 13% beef muscle	32	188 ± 7.0	1.58 ± .07
24% Casein + 4% whole desiccated liver	23	194 ± 6.9	1.60 ± .09
17% Whole desiccated liver + 24% casein	32	208 ± 4.0	1.95 ± .14
21% Beef muscle	20	174 ± 6.3	1.74 ± .09

<sup>a</sup> Protein content based on Kjeldahl N × 6.25

<sup>b</sup> After 150 days of feeding

<sup>c</sup> Supplemented with 1 mg. of biotin

strate that the gain in body weight of the animals fed the various diets for 150 days was essentially the same regardless of the protein supplement or the per cent of protein in the diet. However marked differences in the cholinesterase concentration in different groups were observed. For example supplementation of the basal casein diet with beef muscle or whole desiccated liver elevated the level of the enzymatic activity. Although feeding a diet supplemented with 4% whole desiccated liver

resulted in a significantly increased plasma cholinesterase activity. 13% beef muscle was needed to bring about an equivalent increment. Therefore, whole desiccated liver is believed to be richer than beef muscle in the nutrients responsible for this effect. The increase in the enzymatic activity is not due to the protein content of the diet since increasing the casein to 38% did not elevate the plasma cholinesterase level. Furthermore, it seems unlikely that this increment in enzyme level results from changing the pattern of amino acids in the diet by the supplementation of another protein since the addition of egg albumin to the basal casein diet was ineffective.

The testing procedure thus far employed is adequate to show the difference in plasma cholinesterase levels of rats fed various protein preparations, but requires a long period of feeding and, therefore, a large amount of the test material. Attempts were made to shorten the time necessary to demonstrate this phenomenon. It was found that feeding 65 day old female rats the various test protein preparations for a period of 30 or 60 days resulted in marked differences in plasma cholinesterase. The results of a typical experiment are shown in Table XII. Feeding a 21% beef muscle diet to 65 day old female rats for 30 or 60 days resulted in significantly higher levels of the enzyme than feeding a diet containing 24% or 38% casein. However, increasing the beef muscle of the diet to the 34% level did not further elevate the plasma cholinesterase.

Fractions obtained from whole desiccated liver were assayed with this experimental procedure for their ability to increase the level of plasma cholinesterase. The rats were fed the basal casein diet (24%) to which various liver fractions were added. After 60 days of feeding the plasma cholinesterase was determined. In order that observed differences in feeding the various liver fractions could not be attributed to the differences in the protein or vitamin contents of the diets, an experiment was carried out in which one group of animals was fed a 24% casein plus 17% whole desiccated liver diet and the other group fed a 38% casein diet containing an amount of water soluble vitamins equal to that of the former diet. These data (Table XIII) showed that the plasma cholinesterase level of female rats fed the casein diet supplemented with additional vitamins failed to equal that of animals fed a diet containing whole desiccated liver. Therefore it seems unlikely that the nutrients present in whole desiccated liver responsible for this phenomenon are identical to any of the known water soluble vitamins. In the second experiment 2 fractions obtained from whole liver, namely liver concentrate and liver residue, were assayed for their ability to increase the level of this enzyme. Liver residue and liver concentrate

TABLE VII  
THE EFFECT OF THE INCUBATION OF CASEIN OR BEEF MUSCLE ON THE PLASMA CHOLINESTERASE OF 65 DAY OLD FEMALE RATS

Diets <sup>a</sup>	% Proteins <sup>b</sup>	30 Days of feeding		60 Days of feeding	
		Gain in body weight (gm)	Plasma cholinesterase (RP units/0.3 ml)	Gain in body weight (gm) <sup>c</sup>	Plasma cholinesterase (RP units/0.3 ml)
24% Casein	20	54 ± 7.1	1.22 ± 10	69 ± 7.0	1.29 ± 10
38% Casein	32	45 ± 2.5	1.01 ± 10	63 ± 3.8	1.32 ± 13
21% Beef muscle	20	60 ± 6.7	1.49 ± 0.8	77 ± 7.9	1.70 ± 11
34% Beef muscle	32	51 ± 6.2	1.52 ± 10	76 ± 4.2	1.72 ± 0.5

<sup>a</sup> All diets contained 300 µg of vitamin B<sub>12</sub> per kilogram

<sup>b</sup> Protein content based on Kjeldahl N × 6.25

<sup>c</sup> Six animals per group with initial body weights of 149 ± 3.1



resulted in a significantly increased plasma cholinesterase activity, 13% beef muscle was needed to bring about an equivalent increment. Therefore, whole desiccated liver is believed to be richer than beef muscle in the nutrients responsible for this effect. The increase in the enzymatic activity is not due to the protein content of the diet since increasing the casein to 38% did not elevate the plasma cholinesterase level. Furthermore, it seems unlikely that this increment in enzyme level results from changing the pattern of amino acids in the diet by the supplementation of another protein since the addition of egg albumin to the basal casein diet was ineffective.

The testing procedure thus far employed is adequate to show the difference in plasma cholinesterase levels of rats fed various protein preparations, but requires a long period of feeding and, therefore, a large amount of the test material. Attempts were made to shorten the time necessary to demonstrate this phenomenon. It was found that feeding 65 day old female rats the various test protein preparations for a period of 30 or 60 days resulted in marked differences in plasma cholinesterase. The results of a typical experiment are shown in Table XII. Feeding a 21% beef muscle diet to 65 day old female rats for 30 or 60 days resulted in significantly higher levels of the enzyme than feeding a diet containing 24% or 38% casein. However, increasing the beef muscle of the diet to the 34% level did not further elevate the plasma cholinesterase.

Fractions obtained from whole desiccated liver were assayed with this experimental procedure for their ability to increase the level of plasma cholinesterase. The rats were fed the basal casein diet (24%) to which various liver fractions were added. After 60 days of feeding the plasma cholinesterase was determined. In order that observed differences in feeding the various liver fractions could not be attributed to the differences in the protein or vitamin contents of the diets, an experiment was carried out in which one group of animals was fed a 24% casein plus 17% whole desiccated liver diet and the other group fed a 38% casein diet containing an amount of water soluble vitamins equal to that of the former diet. These data (Table XIII) showed that the plasma cholinesterase level of female rats fed the casein diet supplemented with additional vitamins, failed to equal that of animals fed a diet containing whole desiccated liver. Therefore it seems unlikely that the nutrients present in whole desiccated liver responsible for this phenomenon are identical to any of the known water soluble vitamins. In the second experiment 2 fractions obtained from whole liver, namely liver concentrate and liver residue, were assayed for their ability to increase the level of this enzyme. Liver residue and liver concentrate

TABLE VII  
THE EFFECT OF THE INGESTION OF CASEIN OR BEEF MUSCLE ON THE PLASMA CHOLINESTERASE OF 65 DAY OLD FEMALE RATS

Diets <sup>a</sup>	% Proteins <sup>b</sup>	30 Days of feeding		60 Days of feeding	
		Gain in body weight (gm)	Plasma cholinesterase (RP units/0.3 ml)	Gain in body weight (gm) <sup>c</sup>	Plasma cholinesterase (RP units/0.3 ml)
24% Casein	20	54 ± 7.1	1.22 ± 10	69 ± 7.0	1.29 ± 10
38% Casein	32	45 ± 2.5	1.01 ± 10	63 ± 3.8	1.32 ± 13
21% Beef muscle	20	60 ± 6.7	1.49 ± 0.8	77 ± 7.9	1.70 ± 14
34% Beef muscle	32	51 ± 6.2	1.52 ± 10	76 ± 1.2	1.72 ± 0.5

<sup>a</sup> All diets contained 300 µg of vitamin B<sub>1</sub> per kilogram

<sup>b</sup> Protein content based on Kjeldahl N × 6.25

<sup>c</sup> Six animals per group with initial body weights of 149 ± 3.1

are the materials which are insoluble and soluble, respectively, in hot water after extraction of whole liver. Each 17 gm of whole desiccated liver contains 14 gm of liver residue and 3 gm of liver concentrate.

TABLE XIII  
THE EFFECT OF THE INGESTION OF WHOLE DESICCATED LIVER ON THE PLASMA CHOLINESTERASE OF FEMALE RATS<sup>a</sup>

Diets	% Protein <sup>b</sup>	Initial body weight (gm)	Gain in body weight (gm)	Plasma cholinesterase (RP units/0.3 ml)
38% Casein <sup>c</sup>	32	188 ± 4.5	59 ± 2.0	1.22 ± 0.6
24% Casein + 17% whole desiccated liver	32	186 ± 8.8	59 ± 2.0	1.83 ± 1.1

<sup>a</sup> Six animals per group

<sup>b</sup> Protein content based on Kjeldahl N × 6.25

<sup>c</sup> Additional vitamins added per kilogram of diet: 170 mg riboflavin, 680 mg niacin, 26 mg pyridoxine HCl, 340 mg calcium pantothenate, 2560 mg choline chloride, 34 mg folic acid, 60 mg inositol, 23 mg thiamine, 120 µg vitamin B<sub>12</sub>.

TABLE XIV  
THE EFFECT OF THE INGESTION OF LIVER FRACTIONS ON THE PLASMA CHOLINESTERASE OF FEMALE RATS

Diets	No. of rats	Initial body weight (gm)	Gain in body weight (gm)	Plasma cholinesterase (RP units/0.3 ml)
24% Casein	8	174 ± 5.9	54 ± 4.7	1.38 ± 1.2
24% Casein + 17% WDL <sup>a</sup>	8	181 ± 5.4	49 ± 4.7	1.77 ± 0.8
24% Casein + 3% LC <sup>b</sup>	7	175 ± 7.3	47 ± 7.1	1.77 ± 1.0
24% Casein + 14% LR <sup>c</sup>	7	176 ± 4.7	56 ± 4.8	1.50 ± 0.6

<sup>a</sup> WDL = whole desiccated liver

<sup>b</sup> LC = liver concentrate

<sup>c</sup> LR = liver residue

The results found in Table XIV indicated that feeding a 24% casein diet supplemented with 3% liver concentrate but not 14% liver residue increased the level of plasma cholinesterase to that of animals fed the diets supplemented with 17% whole desiccated liver.

The nature of the nutrients responsible for this phenomenon is as yet unknown. However, since the beef muscle was extracted with benzol and contained little fat by analysis, the active material is probably not a fat soluble component of the diet. In addition, the available data fail to indicate that any of the known water soluble vitamins are responsible for this phenomenon. Although amounts of the inorganic salts known to be required for adequate nutrition were included in all

diets experiments were not carried out to test other metals such as molybdenum which is essential for the synthesis of xanthine oxidase. Thus these data suggest that the ingestion of some as yet unknown factors found in beef muscle and whole desiccated liver results in an increased plasma cholinesterase of young growing female rats.

### B. PROTEIN DEPLETED ADULT RATS

It was of interest to determine whether the increased concentration of plasma cholinesterase which results from the feeding of diets containing beef muscle to young growing female rats could likewise be demonstrated in adult animals. Such attempts were made by first depleting the plasma cholinesterase by feeding a protein free diet and then measuring the rate of regeneration of the enzyme following feeding of diets which contained different dietary protein preparations. Nineteen animals were fed a protein free diet (84% sucrose, 12% mazola oil, 4% salt IV and adequate amounts of the known vitamins) for 45 days. The animals were bled periodically by cardiac puncture and the cholinesterase activity of the plasma determined. The concentration of the enzymatic activity is readily lowered during protein depletion (Table V). Furthermore the concentration of this enzyme appears to reach

TABLE V  
THE EFFECT OF PROTEIN FREE FEEDING ON THE PLASMA CHOLINESTERASE OF FEMALE RATS

Days on diet	No of rats	Plasma cholinesterase <sup>a</sup>
0	19	100
14	6	55 ± 4.1
31	6	45 ± 4.1
40	5	28 ± 6.0
45	19	32 ± 3.3

<sup>a</sup> Expressed as per cent of initial level (172 ± 07)

a plateau on approximately the 40th day of feeding at which time about 33% of the original activity remains. On the 45th day the animals were fed *ad libitum* one of the following diets: 20% casein, 20% casein to which was added 300 µg vitamin B<sub>12</sub> per kilogram and 20% beef muscle. The rats were bled on the 14th and 28th days of the feeding period and the plasma cholinesterase determined. It may be seen (Table VI) that the rate of return of the enzyme activity was greater in the animals fed the diet containing beef muscle. The addition of vitamin B<sub>12</sub> to the casein diet did not affect the rate of repletion of the cholinesterase. Thus it is possible to demonstrate this phenomenon under

are the materials which are insoluble and soluble, respectively, in hot water after extraction of whole liver. Each 17 gm of whole desiccated liver contains 14 gm of liver residue and 3 gm of liver concentrate.

TABLE XIII

THE EFFECT OF THE INGESTION OF WHOLE DESICCATED LIVER ON THE PLASMA CHOLINESTERASE OF FEMALE RATS<sup>a</sup>

Diets	% Protein <sup>b</sup>	Initial body weight (gm)	Gain in body weight (gm)	Plasma cholinesterase (RP units/0.3 ml)
38% Casein <sup>c</sup>	32	188 ± 4.5	59 ± 2.0	1.22 ± .06
24% Casein + 17% whole desiccated liver	32	186 ± 8.8	59 ± 2.0	1.83 ± .11

<sup>a</sup> Six animals per group.

<sup>b</sup> Protein content based on Kjeldahl N × 6.25.

<sup>c</sup> Additional vitamins added per kilogram of diet: 17.0 mg riboflavin, 68.0 mg niacin, 2.6 mg pyridoxine HCl, 34.0 mg calcium pantothenate, 2560 mg choline chloride, 3.4 mg folic acid, 60 mg inositol, 2.3 mg thiamine, 120 µg vitamin B<sub>12</sub>.

TABLE XIV

THE EFFECT OF THE INGESTION OF LIVER FRACTIONS ON THE PLASMA CHOLINESTERASE OF FEMALE RATS

Diets	No. of rats	Initial body weight (gm)	Gain in body weight (gm)	Plasma cholinesterase (RP units/0.3 ml)
24% Casein	6	174 ± 5.9	54 ± 4.7	1.38 ± .12
24% Casein + 17% WDL <sup>a</sup>	6	181 ± 5.4	49 ± 4.7	1.77 ± .08
24% Casein + 3% LC <sup>b</sup>	7	175 ± 7.3	47 ± 7.1	1.77 ± .10
24% Casein + 14% LR <sup>c</sup>	7	176 ± 4.7	56 ± 4.8	1.50 ± .06

<sup>a</sup> WDL = whole desiccated liver.

<sup>b</sup> LC = liver concentrate.

<sup>c</sup> LR = liver residue.

The results found in Table XIV indicated that feeding a 24% casein diet supplemented with 3% liver concentrate but not 14% liver residue, increased the level of plasma cholinesterase to that of animals fed the diets supplemented with 17% whole desiccated liver.

The nature of the nutrients responsible for this phenomenon is as yet, unknown. However, since the beef muscle was extracted with benzol and contained little fat by analysis, the active material is probably not a fat soluble component of the diet. In addition, the available data fail to indicate that any of the known water soluble vitamins are responsible for this phenomenon. Although amounts of the inorganic salts known to be required for adequate nutrition were included in all

diets experiments were not carried out to test other metals such as molybdenum which is essential for the synthesis of xanthine oxidase. Thus these data suggest that the ingestion of some as yet unknown factors found in beef muscle and whole desiccated liver results in an increased plasma cholinesterase of young growing female rats.

## B PROTEIN DEPLETED ADULT RATS

It was of interest to determine whether the increased concentration of plasma cholinesterase which results from the feeding of diets containing beef muscle to young growing female rats could likewise be demonstrated in adult animals. Such attempts were made by first depleting the plasma cholinesterase by feeding a protein free diet and then measuring the rate of regeneration of the enzyme following feeding of diets which contained different dietary protein preparations. Nineteen animals were fed a protein free diet (84% sucrose 12% mazola oil 4% salt IV and adequate amounts of the known vitamins) for 45 days. The animals were bled periodically by cardiac puncture and the cholinesterase activity of the plasma determined. The concentration of the enzymatic activity is readily lowered during protein depletion (Table XV). Furthermore the concentration of this enzyme appears to reach

TABLE XV  
THE EFFECT OF PROTEIN FREE FEEDING ON THE PLASMA CHOLINESTERASE OF FEMALE RATS

Days on diet	No of rats	Plasma cholinesterase <sup>a</sup>
0	19	100
14	8	55 ± 4.1
31	8	45 ± 4.1
40	5	28 ± 8.0
45	19	32 ± 3.3

<sup>a</sup> Expressed as per cent of initial level ( $1.72 \pm .07$ )

a plateau on approximately the 40th day of feeding at which time about 33% of the original activity remains. On the 45th day the animals were fed *ad libitum* one of the following diets: 20% casein 20% casein to which was added 300 µg vitamin B<sub>12</sub> per kilogram and 20% beef muscle. The rats were bled on the 14th and 28th days of the feeding period and the plasma cholinesterase determined. It may be seen (Table XVI) that the rate of return of the enzyme activity was greater in the animals fed the diet containing beef muscle. The addition of vitamin B<sub>12</sub> to the casein diet did not affect the rate of repletion of the cholinesterase. Thus it is possible to demonstrate this phenomenon under

another experimental condition, namely, repletion of the enzymatic activity in mature protein depleted female rats

TABLE XVI

THE EFFECT OF DIETARY PROTEINS ON THE LEVEL OF PLASMA CHOLINESTERASE OF RATS DEPLETED IN PROTEINS

Diet	% Protein <sup>b</sup>	Plasma cholinesterase <sup>a</sup>	
		14th Day	28th Day
24% Casein	20	159 ± 3.1	200 ± 18.4
24% Casein + vitamin B <sub>12</sub>	20	159 ± 9.4	202 ± 18.4
21% Beef muscle	20	194 ± 12.5	238 ± 15.7

<sup>a</sup> Calculated as per cent of the concentration determined prior to protein feeding

<sup>b</sup> Protein content based on Kjeldahl N × 6.25

#### IV THE EFFECT OF DIETARY PROTEINS ON REPLETION OF LIVER PROTEINS

Addis *et al* (1936) Kosterlitz (1947), and Harrison and Long (1945) found that when rats were allowed to fast or were given a protein free diet, the liver suffered a very outstanding loss of proteins. It was, therefore, of interest to determine the effect of various dietary protein preparations on the repletion of liver proteins. Chow *et al* (1948) subjected healthy normal rats weighing approximately 225 gm, to a 48 hour fast during which time the animals were offered water *ad libitum*. Ten of the animals were sacrificed following the period of starvation. The remaining animals were divided into groups of ten each and were then fed test diets containing 40% protein for 4 consecutive days (10 gm diet for the first day, and 8 gm for the next 3 days). It was found that, as a rule, the rats consumed all the rations as scheduled, the few animals which did not were discarded. The composition of the diet was 40% test protein, 22% partially hydrolyzed starch preparation, 8% yeast, 4% salt, 2% cod liver oil and 24% hydrogenated cotton seed oil. Twenty four hours after the last feeding the animals were sacrificed and the nitrogen content of the liver determined by micro Kjeldahl method. Ten animals not subjected to any of these experimental conditions served as controls. Lactalbumin and casein were definitely superior to whole egg or egg white for the regeneration of liver proteins (Table XVII). It is also interesting to note that wheat gluten is not inferior to egg proteins according to this test.

Examination of liver for specific tissue components following periods of depletion have also been carried out. For example, the loss of liver proteins was likewise accompanied by a decrease in the concentration of enzymes such as cathepsin dehydrogenase etc. which could be restored on protein repletion (Miller 1948) Chow *et al* (1949b) re

TABLE XVII  
THE EFFECT OF VARIOUS DIETARY PROTEINS ON THE REGENERATION OF LIVER PROTEIN IN THE RAT

Category of interest	Control	Fasting	Diets fed			
			Egg white	Lactalbumin	Whole egg	Wheat gluten
Mg N per 100 gm body weight	122 ± 1.2	91 ± 1.6	105 ± 1.1	125 ± 1.8	109 ± 1.8	117 ± 1.6
						107 ± 1.1



another experimental condition, namely, repletion of the enzymatic activity in mature protein depleted female rats

TABLE XVI

THE EFFECT OF DIETARY PROTEINS ON THE LEVEL OF PLASMA CHOLINESTERASE OF RATS DEPLETED IN PROTEINS

Diet	% Protein <sup>b</sup>	Plasma cholinesterase <sup>a</sup>	
		14th Day	28th Day
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24% Casein + vitamin B <sub>12</sub>	20	159 $\pm$ 94	202 $\pm$ 18
21% Beef muscle	20	194 $\pm$ 125	238 $\pm$ 15

<sup>a</sup> Calculated as per cent of the concentration determined prior to protein feeding

<sup>b</sup> Protein content based on Kjeldahl N  $\times$  6.25

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Mg N per 100 gm body weight	122 ± 1.2	91 ± 1.6	105 ± 1.1	125 ± 1.8	109 ± 1.8	117 ± 1.6
						107 ± 1.4

ported that severe protein depletion brought about a decrease in the activity of the as yet unidentified nutritional accessory factors. The factor stimulated the growth of *Streptococcus faecalis* Rough in a medium containing adequate amounts of crystalline vitamin B<sub>12</sub>. It also corrected the growth retardation effects of sulfasuxidine in young rats. This nutrient differed from vitamin B<sub>12</sub> chemically in that its microbiological activity could be destroyed by Wilson's purified trypsin (1:280) but did not affect the activity of vitamin B<sub>12</sub>. The destructibility of microbiological activity by the proteolytic enzyme preparation suggested the protein nature of this factor. The microbiological activity in a liver homogenate was markedly increased by some ten or more times if it were allowed to autolyze at 37°C for approximately 48 hours. It was, therefore, of interest to ascertain whether different dietary proteins are equally effective in replenishing this liver factor. Results showed that when depleted rats were repleted with different proteins, the number of microbiological units per gram of fresh liver in the saline extract after incubation differed according to the protein fed (160 ± 4 for casein, 100 ± 6 for wheat gluten, and 60 ± 9 for egg white).

The results indicate, therefore, that the ability of a given dietary protein to stimulate the synthesis of tissue proteins or of a nutritive factor like the one mentioned above is not necessarily correlated with the orthodox growth efficiency tests.

## V. DISCUSSION

The classic studies of Rose (1935), Madden and Whipple (1936) and Frazier *et al.* (1947) demonstrated beyond doubt that synthesis of tissue proteins requires the presence of all the so-called essential amino acids in the diet. The requirements for these acids may vary slightly, however, from one species of animal to another and may also be dependent upon the criterion used to measure it. Furthermore, data now available indicate that these essential amino acids must be available at the site of synthesis at the proper time. Thus, the relative rate at which the essential amino acids are released from dietary protein within the digestive tract also plays a role in tissue protein synthesis.

The important role of the above factors in protein synthesis can be overemphasized. In this communication evidence is presented of the existence of other factors which may influence the rate of tissue protein synthesis. The variety of conditions under which these data have been obtained add support to this thesis. For example, two species of animals (dogs and rats) have been used in these studies. The experiments have been carried out in young growing animals, protein-depleted adult ones, and finally those subjected to complete inanition. The crit-

used to measure protein synthesis have been nitrogen balance, total liver proteins, various plasma proteins analyzed electrophoretically, and lastly, a specific plasma protein viz. cholinesterase. In all the experiments presented, differences attributable to the ingestion of certain dietary protein preparations could not be explained on the basis of differences in the total amount or pattern of the essential amino acids or of differences in the quantity of the other known essential nutrients such as vitamins and minerals. Therefore, these data are offered as evidence that dietary protein preparations contain some as yet unknown accessory factors which stimulate or direct the synthesis of specific tissue proteins. Unfortunately, the relatively small amount of information presently available regarding the chemical nature of these substances does not make it possible to even speculate whether these factors are integral parts of the protein molecules or represent contaminants which are in the dietary protein preparations. Thus, final proof for this hypothesis must await purification and identification of these factors.

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The important role of the above factors in protein synthesis cannot be overemphasized. In this communication evidence is presented for the existence of other factors which may influence the rate of tissue protein synthesis. The variety of conditions under which these data have been obtained add support to this thesis. For example, two species of animals (dogs and rats) have been used in these studies. The experiments have been carried out in young growing animals, protein-depleted adult ones, and finally those subjected to complete inanition. The criteria



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## CHAPTER 7

# The Effect of Proteins and Amino Acids on the Growth of Adult Tissue *in Vitro*

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<sup>1</sup> The research reported here was completed previous to 1949 and was aided by

## I INTRODUCTION

### A TISSUE CULTURES AS A MEANS FOR STUDYING NUTRITIONAL REQUIREMENTS OF ADULT CELLS

This chapter deals with the nutritional requirements of adult cells in tissue culture. It should be pointed out that most tissue culture work in the past has dealt with embryonic tissue, rather than with adult tissue. This is true of the early work on chick embryo tissue in the laboratories of Carrel, of Fischer, and of many others. The role of embryo extract in cultures of embryo tissue has been given considerable attention. Developments have been reviewed by Morgan (1950) and by Waymouth (1954).

It has been our impression that the requirements of adult cells differ from those of the embryo cell. Embryo extract is foreign to adult tissue and while stimulating, is not needed by it (Parshley *et al.*, 1953, Lasfargues, 1956). On the other hand, the A Factor (Simms and Stillman 1937c), which is produced by leucocytes and is found in blood plasma, is essential for the life of adult cells—but not for embryo cells.

In recent years strains of adult tissue including the "L" strain (of adult mouse subcutaneous connective tissue) from Earle and his co-workers laboratory (1943) have been used for nutritional experiments by Earle and also by Healy *et al.* (1955), by Eagle (1955a,b), and by Waymouth (1956). A discussion of long term cultivation of adult cells can be found in the reports of the Tissue Culture Conference of 1956.

As will be shown later in this chapter in overcoming the dormancy of adult aorta tissue and in supporting subsequent growth embryo extract is inferior to extracts of adult spleen and heart.

The nutritional requirements of different adult cell types in the same animal are not the same (Parshley and Simms, 1950). This confirms earlier work on embryo cells by Baker and Carrel (see Morgan, 1950). Nutritional requirements may also vary with the species and with the state of physiological activity of the cells.

### B STUDIES ON ADULT CHICKEN AORTA FIBROBLASTS

For a number of years the authors of this chapter studied the conditions which were favorable for the growth of adult tissues *in vitro* in order to determine what substances are necessary for the maintenance of adult cell life and what substances serve to overcome the dormancy

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of normal adult tissue. For this purpose special techniques were developed for making quantitative determination of the stimulating or inhibitory effect of any given substance on adult tissues *in vitro*. The methods were applied during the second World War to the testing of materials which might be used to promote wound healing. Some of those observations will be reported here.

Several hundred substances have been tested for their effect on the growth of adult tissues *in vitro*. These include proteins, digested proteins, amino acids, plant materials, vitamins, hormones, enzymes, tissue extracts, sulfur compounds, and various other organic and inorganic compounds, including antibacterial agents and ointment bases.

### C MAINTENANCE VERSUS DORMIANCY—ADULT TISSUE INHIBITOR

It should be explained that by maintenance we mean the nutritional requirements necessary to keep a cell alive and functioning—without cell division. But when we “overcome the dormancy” of adult tissue we must not only maintain the cell in a living state, but we must also eliminate or counteract the “adult tissue inhibitor” which is present in adult tissues (Simms and Stillman 1937b) and is even present in cultures of adult cells. This inhibitor protects adults from unrestrained growth or malignancy. It is a highly important material.

## II METHODS OF OBTAINING DATA

A brief summary of the technique follows. This technique was developed over a period of years (Simms and Stillman 1937a, b, c, d, and e; Simms and Sanders 1942).

### A TISSUES

Adult chicken aorta and adult chicken skin were used for most of the experiments reported here. The aorta cultures produced a clearly distinguishable growth of new fibroblasts. The adult chicken skin was cultured in a special medium which gave sheets of epithelial cells relatively free from fibroblasts.

### B CULTURE MEDIA

The media consisted of chicken plasma diluted with two parts of a suitable “diluting solution” (see below). No heparin or other anti-coagulant was used.

### C COMPOSITION OF PHYSIOLOGICAL SOLUTIONS AND DILUTING SOLUTIONS (TABLE I)

The composition of the solutions which were used for bathing or storing tissues and for diluting the culture media are given in Table I.

TABLE I  
PHYSIOLOGICAL AND DILUTING SOLUTIONS USED IN CULTIVATION OF ADULT TISSUES

Constituents	MW	X6 Solution (pH 7.5)		Z16 Solution (pH 6.9)		Z2 Solution (pH 7.4)	
		(Mixed with 1/2 part plasma for adult aorta fibro- blasts)	(Mixed with 1/2 part plasma for adult skin epi- thelial cells)	(Solution for gen- eral washing and temporary storage)			
		gm per liter	gm per liter	gm per liter	mmM per liter	mmM per liter	mmM per liter
First mother solution <sup>a</sup>							
NaCl	58.5	8.00	137	6.80	116.4	8.00	137
KCl	74.5	0.20	27	0.17	2.3	0.20	27
CaCl <sub>2</sub> 2H <sub>2</sub> O	147	0.147	1.0	0	0.85	0.147	1.0
MgCl <sub>2</sub> 6H <sub>2</sub> O	203	0.203	1.0	0.173	4.7	0.203	1.0
Dextrose	180	0	0	0.85	0.21	0	0
Phenol red	354	0	0	0.075 <sup>b</sup>		1.00	5.5
Second mother solution							
Dextrose	180	1.00	5.5	0	0	0	1.2
Phenol red	354	0.05 <sup>c</sup>	0.14 <sup>c</sup>	0	0	0.10	0.15
NaHCO <sub>3</sub>	84	1.01	12.0	0	0	0.021	1.35
NaH <sub>2</sub> PO <sub>4</sub>	138	0	0	0	9.0	0.190	
Na <sub>2</sub> HPO <sub>4</sub>	142	0.213	1.5	1.28	22.5	0	
Aspartic acid	133	0	0	3.00	19.2	0	
NaOH solution 0.1 M	40	0	0	0.75	3.5	0	
Sulfamethazine	214	0	0				

<sup>a</sup> Two mother solutions are made up twenty times the above concentrations After sterilization two equal volumes are diluted with eighteen volumes of sterile water as previously described (Simms and Sanders 1912)

<sup>b</sup> Plus 0.1 M N<sub>2</sub>O<sub>4</sub> as necessary

<sup>c</sup> Plus concentration of phenol red (0.005%) is used for observing the pH when the volume is 1 or 2 ml

<sup>d</sup> For larger volumes

The X  
aorta fibro  
as a phy  
as a d  
The  
with p  
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incl  
ad  
ts

The "X6 Solution" was developed for the cultivation of adult chicken aorta fibroblasts (Simms and Sanders 1942) It may be used by itself as a physiological solution for bathing or storing tissue It is used also as a "diluting solution" for diluting plasma or serum ultrafiltrate

The "Z16 Solution" is used only as a diluting solution When used with plasma it gives a medium favorable for the growth of adult skin epithelial cells It contains neither bicarbonate nor calcium, but has a high concentration of phosphate It will be noted that aspartic acid is included because of its growth stimulating action Sulfamethazine was added for its antibacterial effect

The "Z2 Solution" is used for washing or for temporary storage of tissues

#### D SERUM ULTRAFILTRATE

Serum ultrafiltrate contains a growth stimulating agent which we call the "A factor" which is essential for adult cell maintenance and for the growth of adult tissues It is used in fluid media diluted with a suitable physiological or diluting solution (Simms and Stillman 1937c; Simms and Sanders 1942) Serum ultrafiltrate must always be kept under an atmosphere containing about 5% CO<sub>2</sub> Otherwise the activity of the A factor is immediately lost due to the change in pH Ultrafiltrate is not species specific

#### E METHODS OF TREATMENT OF TISSUES WITH TEST SUBSTANCES

We have two methods for testing the effect of an agent on the growth of adult tissue the "fluid medium method" and the "plasma medium method" In the fluid medium method the tissue was given a preliminary incubation at 37 C for 3 days in a suitable fluid medium containing the test substance after which it was planted in a plasma clot (without the test substance) and the subsequent growth was evaluated daily This was the more satisfactory method for aorta fibroblast tests and was used almost invariably for them

The fluid medium consisted of X6 solution or other physiological solution or of serum ultrafiltrate diluted with two parts of X6 or other diluting solution In general when stimulating substances were tested, a nonstimulating medium was used But when toxic or inhibitory substances were tested a stimulating medium (containing serum ultrafiltrate) was used In each case the subsequent growth was compared with control solutions containing no test substances

In the "plasma medium method" the fresh tissue was planted directly in a plasma medium containing the test substances This method was used in tests on skin epithelial cells When agents were insoluble in water

that were stimulating are placed in column 4 *inert* in column 5, and so on, according to the criteria indicated in the tabulation

Category		Relative growth rating	
		over 125	— third column
Stimulating	=	125 to 75	— fourth column
Inert	=	75 to 25	— last column
Inhibitory	=	25 to 1	— last column
Toxic	=	0	— last column
Lethal	=		

The type of tissue or cell is indicated by a symbol (see below) This is followed by the relative growth rating (100 times the ratio of the growth rating with the test substance, divided by that of the control solution)

## B ABBREVIATIONS AND SYMBOLS USED IN THE TABLES

### 1 Type of Tissue and Cells

- A = aorta fibroblasts  
 F = skin fibroblasts  
 E = skin epithelial cells  
 Thy = thyroid epithelial cells

### 2 Composition of Fluid Media

In tests using a fluid medium the agent was added to the designated fluid medium in which the tissue was incubated for 3 days before it was planted in a plasma clot (not containing the agent)

- UF = Serum ultrafiltrate (diluted with two or nine parts of physiological solution and designated "UF/3" or "UF/10" respectively)

- D UF = Decarbonated serum ultrafiltrate (diluted)  
 S = Serum (usually diluted with three parts of physiological solution and designated "S/4")

- λ6 = A special physiological or diluting solution (see Table I) which is favorable for adult fibroblasts

- Z16 = A special diluting solution (see Table I) favorable for adult skin epithelial cells

- Z8 = A special diluting solution similar to Z16 but containing one third more phosphate—but no aspartic acid

- Ty = Tyrode solution (not used in this laboratory after March 1937 because it has been replaced by λ6 which is more favorable for adult fibroblast growth)

### 3 Solid Medium

In these tests the agent was added to the plasma clot in which tissue was planted directly

Pl = Plasma medium Blood plasma diluted with two parts of "X6" physiological solution for aorta tissue or with "Z8" or "Z16" solution for skin tissue

### 4 Physical State of Substance in Medium

When no symbol appears the agent was in solution

G = The agent formed a gel

D = The agent was dispersed as a suspension or an emulsion

P = The agent was poorly soluble and settled as a precipitate in whole or in part

S = The agent was insoluble and was floated on the surface

## IV EFFECT OF PROTEINS PHYSIOLOGICAL MATERIALS, AND OTHER SUBSTANCES ON GROWTH

### A BLOOD AND TISSUE CONSTITUENTS INCLUDING HORMONES (TABLE II)

Several constituents of blood are important for growth and maintenance of adult cells *in vitro*. One of these the A Factor (Simms and Stillman 1937c; Simms and Sanders 1942) appears to be indispensable to the maintenance of adult cells. It is present in plasma and serum. Serum ultrafiltrate contains the A Factor and is free from proteins\*. This is used as a basis for a standard medium for the maintenance of adult cells. However serum ultrafiltrate alone is not satisfactory for the initiation of growth. For this reason plasma proteins were usually added to the tissue culture medium.

A material contained in the red cells was highly stimulating to the growth of both fibroblasts and epithelial cells when present in a very low concentration. The active substance cannot be hemoglobin for both hemoglobin and hemin were inert when tested in purified form. Red cell extract was included in certain of our media. An extract of leucocytes was also very stimulating and since this is the only agent which has served as a substitute for serum ultrafiltrate in the maintenance of adult cells over a period of time we have assumed that the A Factor is produced by leucocytes. An extract made from blood platelets was also found to improve the growth of adult fibroblasts.

\* The serum ultrafiltrate was obtained from Microbiological Associates, Bethesda, Maryland. Unfortunately serum ultrafiltrate must be kept and used under an atmosphere containing 3 to 5% CO<sub>2</sub>. Otherwise the loss of CO<sub>2</sub> causes a rise in pH with immediate complete destruction of the A Factor.



TABLE II  
BLOOD AND TISSUE CONSTITUENTS INCLUDING HORMONES

Substance	Concentration (%)	Media	Stimulating	Inert	Inhibitory toxic or lethal
Lymph dog	33	Ty	A 700		
	11	Ty	A 300		
Plasma, chicken	33		Used for basic culture medium		
Serum chicken	33	Ty	A 530		
	25	Ty	A 460		
	10	Ty	A 135		
Serum dog	33	Ty	A 500		
Serum human fetal	15	XG	A 500		
Serum human adult	33	Ty	A 500		
Serum ultrafiltrate dog	100	Ty control	A 660		
	33	Ty	A 725		
Serum ultrafiltrate ox	100	XG control	A 675		
	33	XG	A 790		
	11	XG	A 450		
	4	XG	A 210		
Serum ultrafiltrate rabbit	100	Ty control	A 350		
Adrenal whole extract chicken	10	UF/3		A 117	
Egg white chicken	5	XG	A 506		
	5	Pl/3	E 270		

TAB I F II (Continued)

Substance	Concentration (%)	Media	Stimulating	Inert	Inhibitory toxic or lethal
Egg white chicken (dried)	0.12	UF/10	A 270		
Embryo extract bovine	5	\6	A 360		
Embryo extract chicken	33	S/3		A 110	
	33	UF/3	A 175		
	25	Ty		A 117	
Heart extract chicken	10	Pl/3	■ 126		
			F 400		
	10	\6	A 360		
	10	UF/3	A 200		
	4	UF/36	A (300)		
Heart extract chicken CO <sub>2</sub> ppt fraction	4	UF/36	A (200)		
Heart extract, chicken super natant fluid fraction	4	UF/36	A (300)		
Estrone (ether prep n)	15 ru/ml				
	0.0004	\6			A 49
Lymph node extract, chicken	3	\6	A (800)		
Lymph node extract leukemic mouse	2	\6	A (125)		
Lymph node suspension leu kemic mouse	2	\6	A (250)		
Leucocyte extract, chicken	5	Ty	A (500)		
Leucocyte suspension chicken	2	Ty	A (800)		
Anterior pituitary hormone	0.015 u/ml	S/4	A (260)		

TABLE II (Continued)

Substance	Concentration (%)	Media	Stimulating	Inert	Inhibitory toxic or lethal
Posterior pituitary ox	<1.0	XG	A 155		A 32
Platelet extract (crude) human	<1.0	UF/10			
Red cell extract chicken from laked washed cells	0.5	UF/3	A 133		
	0.1	Ty	A (200)		
	5.0	UF/3 or PI/3	III 215 (inconsistent)		
Red cell suspension chicken	0.3	Ty	A (500)		
Hemoglobin		XG			A 0
Hemun		XG	A (100)		
Thyroid extract chicken	10.0	Ty	A 260		
	4.0	UF/3	A 152		
	4.0	PI/3	F 250		
Thyroid pig (crude dried)	<0.3	PI/3			E 57
					Thy 66
					E 4
Thyroglobulin	<0.3	XG	A 185		
	0.3	XG	A 160		
Thyrovine	0.3	UF/3			E 3
	0.1				
	(1.25mM)	Ty			
Chromatin solution	1.0	UF/3	A 260 (inconsistent)		A 40

Most of the hormones had little effect on adult cells. A fresh thyroid extract, crude dried thyroid and thyroglobulin were stimulating to fibroblasts but inhibited the growth of skin epithelium and thyroid epithelium. Thyroxine has no stimulating effect. Anterior pituitary extract (a standardized aqueous preparation) greatly stimulated the growth of aorta fibroblasts. Other hormones including crude posterior pituitary extract and a fresh adrenal extract had slight if any stimulating effect. A purified preparation of estrone was inhibitory to fibroblasts.

Extracts from some adult organs stimulated a growth of new cells when added to explanted aorta. Whole heart extract and fractions of it were extremely stimulating to both fibroblasts and epithelial cells. On the other hand, chicken embryo extract was inconsistent in its action on adult tissue. We found it to be unsatisfactory for use in adult tissue culture. Furthermore, it is a medium unnatural for adult cells. Doljansk *et al.* (1942) state that embryo extract is far less stimulating for adult fibroblasts and epithelial cells than extracts of adult brain, heart, and smooth muscle. However, for embryo tissues, Davidson and Waymouth (1945) find sheep embryo extract superior to adult tissue extracts.

Egg white (both fresh and dried) contains a substance which is very stimulating to the growth of both fibroblasts and epithelial cells. It has been used as a substitute for serum in one of our basic culture media but does not give as consistently good results. It appears also to contain the factor which produces cohesiveness of cells (Simms 1936).

## II PROTEINS AND DIGESTED PROTEINS (TABLE III)

Hemoglobin and certain tissue proteins have already been discussed under "blood and tissue constituents" (Table II).

The importance of proteins and their split products to embryo cell growth was observed by the Lewises (1911) who found that the addition of amino acid and polypeptide mixtures to their media enhanced the growth of cells. Carrel and Baker (1926) observed that proteoses and peptones from the peptic digestion of proteins were superior to lower split products as growth stimulants. Baker and Carrel (1928) obtained embryo cell stimulation by hydrolyzates of egg albumin, casein, edestin and fibrin. However, the addition of glycine improved the stimulation. Willmer and Kendal (1932) found that embryo cells require plasma or embryo extract in addition to the proteoses.

Adult cells as seen in Table III were stimulated by certain of the plasma proteins especially thrombin, euglobulin, gamma globulin and fibrinogen.<sup>3</sup> Casein after digestion by pepsin had a marked growth

<sup>3</sup> These serum proteins all contain lipofrogens and are not suitable for tissue cultures where the prevention of fat granules is desired.

TABLE III  
PROTEINS AND DIGESTED PROTEINS

Substance	Concentration (%)	Media	Stimulating	Inert	Inhibitory toxic or lethal
Serum euglobulin ox	10	UF/3	E 238 F 148		
Serum gamma globulin human	33	Pl/3		F 100	E 46
	0.5	UF/3	E 430		
	0.25	UF/3	F 156 E 247 F 430		
	0.125	UF/3	F 302	E 92	
	0.5	Pl/3		F 83	
Cohn's plasma fractions human					
Albumin (fraction V)	0.25	Pl/3	E 132		
Fraction I (mostly fibrinogen)	<0.5	Pl/3			
	<0.25	UF/3	A 116	E 91 F 105	
Fraction IV (mostly alpha globulin)	0.5	Pl/3			
	0.5	UF/10		E 84 F 119	
	0.25	UF/10		A 95 A 117	

TABLE III (Continued)

Substance	Concentration (%)	Media	Stimulating	Inert	Inhibitory toxic or lethal
Gamma globulin	0.5	P1/3		E 91 F 90	
	0.25	P1/3		E 82	
	0.5	UF/3	E 212		
	0.25	UF/10	A 112		
	0.5			A 92	
Gamma globulin with penicillin (170 u/ml)	0.25	UF/10			A 71
Thrombin	0.5	P1/3	F 175	E 112	
	0.5	UF/10	A 200		
	0.25	UF/10	A 274		
Plasma fibrinogen chicken	33	S/10	A 184		
	33	S/3	E 178		
	33	P1/3	E 160		
Casein digested by pepsin	17	P1/3	E 216 F 450		
Lung protein	0.25	X6		A 80	
Placental protein	0.25	X6			A 0

promoting effect on adult aorta fibroblasts and was also very stimulating to the growth of skin epithelium. Hanks (1955) reported that the euglobulin fraction of serum contains growth stimulants. Serum albumin was not particularly stimulating in our tests although Waymouth (1955) reported that albumin (Armour fraction V) added to a simple medium enhanced growth of L strain of adult cells.

The growth promoting properties of tissue extracts have been ascribed to the nucleoproteins by Fischer (1946) and by Davidson and Waymouth (1945).

### C AMINO ACIDS (TABLE IV)

Most of the amino acids which were tested with the exception of arginine and an impure preparation of glycine, were found to have a mild stimulating effect when added to a medium consisting of a physiological solution but gave no added stimulation when they were tested in serum ultrafiltrate. The small concentration of amino acids present in serum ultrafiltrate (only 1.4 millimolar) should not account for this difference. It seems likely that the stimulating action of most amino acids is inconspicuous in comparison with that of the A Factor which is present in the ultrafiltrate. However aspartic acid, asparagine, and proline greatly increased the rate of growth of skin epithelial cells when added to a plasma medium. Aspartic acid and proline had some stimulating effect on fibroblasts in the same medium.

Efforts have been made by other workers to concoct synthetic tissue culture media free from proteins or other substances of unknown chemical structure. Such media have contained amino acids in addition to salts, glucose and vitamins (Evans and associates in Earle's laboratory, 1956a,b). The amino acid composition of such synthetic media has varied widely (White 1946; Morgan, *et al.* 1950), Eagle 1955, Healy *et al.* 1955). Media having no other source of nitrogen than the amino acids can be sufficient for cell survival. However, protein molecules appear to be needed for cell division. Amino acid mixtures are invariably inferior to protein digests as growth stimulants.

The dialysis of plasma, serum and tissue extracts was found (Fischer and co-workers 1946-1948) to leave a residue inadequate for growth. They concluded that this was due to a loss of amino acids and other nutrients—especially cystine. White (1949) and Harris (1952) obtained more favorable results in their tests with dialyzed serum on embryo cultures. However it has been our experience that adult tissues require the A Factor that is removed by dialysis (Summs and Stillman 1937c, Summs and Sanders 1942).

Baker and Carrel (1926) considered that amino acids stimulate embryo cell division and migration. Their results with the addition of glycine to digested proteins have already been mentioned. On the other hand Vogelwar and Erlichman (1936) found that glycine inhibited human thyroid fibroblasts while Burrows and Neyman (1917) reported toxic action on embryo cells of a number of amino acids. Carrel and Ebeling (1924) stated that 20-50 mg. of glycine per 100 ml. had no effect on growth but accelerated migration.

Eagle (1955) reported that 13 amino acids were essential for growth of two cell strains. Lark's "L" strain of fibroblasts from the subcutaneous connective tissue of an adult mouse and Gey's HeLa strain isolated from a human uterine carcinoma. The amino acids listed all L isomeric were arginine, cysteine, glutamine, histidine, isoleucine, lysine, methionine, phenylalanine, threonine, tryptophan, tyrosine, and valine. It is of interest that although the author states that the amino acid requirements of these two extremely different cell types proved remarkably similar, there was a marked difference in concentration for optimum growth. Glutamine is considered to play an essential metabolic role. Fischer and co-workers (1946-1948) had stressed earlier the importance of glutamine as a growth stimulant. Waymouth (1955) observed also that growth was improved by the addition of glutamine, hydroxanthine to a modification of the semi-synthetic medium of Wilson *et al.* (1942) in which Whites' amino acid mixture was substituted for Witte's peptone. Greatly increasing the phosphate and  $\text{NaHCO}_3$  further improved the medium. Glutamic acid at any concentration failed to permit growth of the "L" strain of fibroblasts. It was effective for the HeLa cell in concentrations ten to twenty times that of glutamine.

Eagle (1955) noted that a medium containing these 13 amino acids plus 7 vitamins found to be essential and glucose did not permit growth unless a small amount of serum protein (serum 1-5%) was present. Attempts to analyze the function of the serum residue suggested to the author that trace elements bound to the protein might be of importance. Human plasma fractions I, II, and III were inert and IV and V only weakly active separately or in combination. Exhaustively dialyzed serum was inactive while fractions obtained by simple  $(\text{NH}_4)_2\text{SO}_4$  salting out followed by 24 hour dialysis were all more or less equally active. More recently Lockart and Eagle (1959) have reported that this minimum growth medium supplemented with dialyzed serum which supported the propagation of heavily inoculated cultures of cell strains was not satisfactory for regular or optimum growth of small numbers of cells or cloning from single cells. The deficiency was overcome by the addition of the 7 nonessential amino acids (alanine as



TABLE IV  
AMINO ACIDS

Substance	Concentration	Media	Stimulating	Inert	Inhibitory toxic or lethal
<i>D</i> l Alanine	0.07% ( 7.5 mM)	X6	A 195		
	0.13% (15 mM)	UF/10		A 94	
	0.07% ( 7.5 mM)	Pl/3	F 240	E 89	
Arginine	0.15% ( 7.5 mM)	X6			A 0
	0.15% ( 7.5 mM)	Pl/3			E 33
	0.15% ( 7.5 mM)	S/3			A 60
	0.3 % (15 mM)	UF/10			A 54
<i>L</i> Asparagine	0.1 % ( 7.5 mM)	X6	A 266		
	0.2 % (15 mM)	UF/10		A 87	
	0.2 % (15 mM)	Pl/3	E 440 F 375		
<i>L</i> Aspartic acid (Na salt)	0.2 % (15 mM)	UF/3	E 440		
	0.13% (10 mM)	Pl/3	F 375	A 88	
	0.1 % ( 7.5 mM)	Pl/3	E 132 E 164	F 68 F 100	
	0.09% ( 7.5 mM)	X6			A 0
Cysteine HCl	0.18% (15 mM)	UF/10			A 37
	0.09% ( 7.5 mM)	Pl/3	F 290	E 83	
<i>d</i> Glutamic acid (Na salt)	0.11% ( 7.5 mM)	X6	A 254		
	0.22% (15 mM)	UF/10			
	0.22% (15 mM)	Pl/3		A 82 E >100	F 50

TABLE IV (Continued)

Substance	Concentration	Media	Stimulating	Inert	Inhibitory toxic or lethal
Glycine (impure)	0.15% (10 mM)	Pl/3		F 112	
	0.11% (7.5 mM)	Pl/3		F 79	F 35
	0.1 % (7.5 mM)	Pl/3			E 51 F 75 A 21
l Leucine	0.1 % (7.5 mM)	UF/10		A 97	
	0.1 % (7.5 mM)	Pl/3		E 119	
	0.15% (10 mM)	UF/3			E 63
l Proline	0.08% (7.5 mM)	X6	A 232		
	0.17% (15 mM)	UF/10		A 100	
	0.17% (15 mM)	UF/3	E >200	F 105	
	0.1 % (10 mM)	Pl/3	E 152	F 109	
	0.08% (7.5 mM)	Pl/3	E 143 F 188		
l Hydroxyproline	0.1 % (7.5 mM)	X6	A 268		
	0.2 % (15 mM)	UF/10		A 74	
	0.2 % (15 mM)	Pl/3	E 140		
	0.1 % (7.5 mM)	Pl/3		E 125	
d l Serine	0.08% (7.5 mM)	X6	A 169		
	0.16% (15 mM)	UF/10		A 79	
	0.08% (7.5 mM)	Pl/3			E 72

partic acid, asparagine, glycine, glutamic acid, proline and serine) In most experiments serine alone was sufficient These authors conclude also that specific metabolites may be required for the cultivation of mammalian cells isolated directly from the animal which are not necessary for the propagation of established cell lines

Furthermore, Waymouth's (1956) serum free nutrient for "L" strain mouse fibroblasts contains (in addition to salts sugars, vitamins 0.5% Difco Bacto Peptone, and 0.05% Armour's bovine albumin fraction V) the amino acids listed by Eagle as essential, but in different concentrations L aspartic acid, L glutamic acid, glycine and L-proline were also present and the total amino acid content was ten times that in Eagle's medium (in which serum is present) The presence of peptone was of paramount importance It was suggested by Winnick and Winnick (1953) that the peptides themselves may be assimilated by the cells, and perhaps even whole protein molecules

In cultures of chick tissues there was substantial utilization of proteins in the presence of simultaneous amino acid uptake and incorporation (Francis and Winnick 1953) Harris (1958) cites a number of experimental studies which indicate the penetration of cells by proteins without preliminary breakdown and evidence that in chick embryo tissue cultures the proteins are the preferred precursors of chick embryo tissues He felt that a minimal synthesis of protein from amino acid precursors may be necessary for the utilization of media proteins by direct incorporation Thus a deficiency of lysine or other essential amino acid may block both

Workers in Earle's laboratory have kept alive for several years strains of cells from adult mouse monkey, and human tissue in the protein free media of known chemical composition mentioned above (Evans, 1957) However the addition of 10% gross globulin residue or 0.25% of horse serum greatly increased proliferation and improved the condition of the cells These workers reported that all the protein fractions of horse serum possessed growth stimulating properties inversely proportional to the amount of manipulation required to separate the fractions They suggested that the growth promoting factor of serum is nonspecific However ovalbumin was far less stimulating for these mammalian cell strains than fractions of horse serum Evans in a discussion of the factor or factors important to the growth of animal cells associated with serum proposed that the serum proteins may provide additional amounts of an agent present in growth limiting amounts in synthetic media, or may have a protective action against toxic agents present such as traces of heavy metals The chemically defined media in use today although they have been satisfactory for the continuous proliferation of some cell

strains have proved inadequate for *in vitro* maintenance of highly specialized functioning cells. There is also mounting evidence that the cells of stable cell strains are not representative of cells freshly isolated from the animal.

#### D VITAMINS (TABLE V)

The only vitamin we found to have appreciable stimulating action on adult tissue was ascorbic acid. This stimulated both fibroblasts and epithelial cells. However its effect was not sufficiently beneficial to warrant its incorporation in our standard media.

Working with embryo tissues Chambers and Cameron (1943) concluded that ascorbic acid is essential to epithelial growth and should be added to cultures in Tyrodes solution or scorbutic plasma. Von Jency and Toro (1936) claimed that ascorbic acid aided argyrophil fiber formation.

As for other vitamins tests on embryo cultures by Gordonoff and Ludwig (1937) indicate that Vitamins A, B<sub>1</sub> and B<sub>2</sub> stimulate growth whereas deficiency or excess of C, D and E had no effect. Baker (1935) found no effect of carotene on embryo fibroblasts. However she found stimulation from a small concentration of serum which had been saturated with Vitamin A (from halibut liver oil). Greater concentrations proved toxic. Our tests with similar material failed to stimulate adult fibroblasts.

Yeast extract reported by Heaton (1929) to inhibit the growth of fibroblasts but not of epithelium had the opposite effect in our cultures.

#### E ENZYMES (TABLE VI)

The stimulating action on fibroblasts of trypsin and papain was very marked when used to partially digest fresh adult aorta tissue. The addition of sulfadiazine appears to increase the stimulating effect. However conditions had to be so carefully controlled (to prevent over digestion or underdigestion) that it was not practical as a routine procedure. Previous work (Simms and Stillman 1937b) showed that the proteolytic digestion removed an inhibitory material contained in adult tissue. Digestion of skin inhibited the growth of epithelial cells.

#### F OTHER ORGANIC SUBSTANCES (TABLE VII)

This group includes several substances of interest in relation to wound healing. The toxicity of rubber extracts presents a problem for tissue culture workers since it necessitates that rubber stoppers should never come in contact with solutions.

TABLE V  
VITAMINS

Substance	Concentration	Media	Stimulating	Inert	Inhibitory toxic, or lethal
p Aminobenzoic acid	0.2% (15 mM)	UF/10			A 67
	0.2% (15 mM)	X6			A 5
Ascorbic acid	0.01% (0.6 mM)	Pl/3		F 120 E 99	
	0.01% (0.6 mM)	S/4	E 150 F 120		
	0.01% (0.6 mM)	UF/3	E 190		F 110
	0.01% (0.6 mM)	UF/10	A 127		
Biotin	0.002 $\gamma$ per ml	X6	A >125		
	0.02 $\gamma$	X6		A 100	
Cod liver oil concentrate (Vitamins A and D) extracted with S/100	1.0% 1.0%	S/3 UF/3		A 98	A 33
Cod liver oil (5 purified fractions containing Vit A)	S 1.0%	floats on UF/3 or Pl/3			
	S 0.25%	floats on UF/3 or Pl/3			EF 0
Nicotinamide	0.2% (15 mM)	m UF/10		A 103	EF 0
Sodium pantothenate	1.8% (7.5 mM)	m X6			A 10
Yeast extract (Nutoplex)	0.2%	in S/4		F 128	E 17

### G SULFUR COMPOUNDS (TABLE VIII)

Most of the SH compounds recommended as stimulating proved to be toxic to the growth of adult cells *in vitro*. Cysteine and glutathione had some stimulating effect on fibroblasts. Hueper and Russell (1933) found no stimulation with these substances when the medium was otherwise adequate. Baker (1929) reported stimulation with glutathione especially when hemoglobin was also present.

Our tests with *p*-thiocresol in the concentration recommended by Reimann (1930, 1931) showed complete toxicity for both fibroblasts and epithelial cells.

The action of thiouracil and thiourea on epithelial cells is of interest. These substances are said to produce thyroid hyperplasia (Ingle 1945). Thiouracil stimulates the growth of thyroid epithelium and to some extent skin epithelium. Thiourea in the same concentration also stimulates the growth of skin epithelium but on the other hand inhibits the growth of thyroid epithelium.

### H PLANT MATERIALS (TABLE IX)

These substances are of interest in relation to wound healing [Parshley and Simms (1950)].

### I INORGANIC COMPOUNDS (TABLE X)

Iron, aluminum, and barium salts in small concentrations improved the growth of adult fibroblasts. Ferrous chloride was stimulating also to the growth of skin epithelium. The addition of iron salts to our plasma medium has not proved advantageous. A precipitate was formed and the stimulating action was inconsistent.

Lead, cadmium, copper, and zinc salts as well as chromates were lethal in our tests, while tin salts were inert. These observations are of interest in the choice of metals to be used in contact with living tissues.

The tests on "Calgon" indicate that it may be used safely for washing glassware. Soaps have the disadvantage that they produce fat granules (see Simms *et al.* 1947). Calgonite is said to contain soap.

### V SUMMARY OF SUBSTANCES FAVORING ADULT CELL GROWTH (TABLE XI)

The word "stimulation" is used in this chapter to include any process which results in an increase in the number of new visible cells greater than in the corresponding control culture. Conceivably the stimulating action of a substance may affect the tissue by one or more of the following processes:

TABLE VI  
ENZYMES<sup>a</sup>

Substance	Concentration (%)	Media	Stimulating	Inert	Inhibitory toxic or lethal
Papain (2 hr at 37 C)	0.1	UF/3	A 184		
	0.001	UF/3	A 136		
Pepsin (18 hr at 20 C)	0.1	UF/3			A 5 EF 0
Procyanase (18 hr at 20 C)	0.01	UF/3			A 57 F 60 E 9 E 50 A 55
Trypsin (2 to 4 hr at 37 C)	0.001	UF/3			
	0.2	UF/3			
	0.2	X6			
Trypsin (2 hr at 37 C)	<0.1	UF/3		F 75	
	0.001	UF/3		A 91	
Trypsin (3 hr at 37 C)	<0.1	UF/3	A 142		
Trypsin (with 0.1% Sulfadiazine 3 hr at 37 C)	<0.1	UF/3	A 129	A 115	
Trypsin (20 hr at 37 C)	<0.1	UF/3	A 230		
Trypsin (18 hr at 20 C)	<0.1	UF/3	A 181		
Trypsin (with 0.05% Sulfadiazine 18 hr at 20 C)	<0.1	UF/3	A 170		
Trypsin (18 hr at 1 C)	0.1	UF/3	F 217		E 15
	0.1	Ty	A 265		
	0.5	Ty			A 0

<sup>a</sup> These were tested on skin tissue at pH 6.9 or on aorta tissue at pH 7.4

TABLE VI (Continued)

Substance	Concentration (%)	Media	Stimulating	Inert	Inhibitory toxic or lethal
Trypsin (18 hr at 4 C) (Cont)	0.1	UF/1	A 316		
	0.06	UF/1	A 255		
	0.05	UF/1	A 191		
Lysozyme preparation (Meyer)	0.01	S/3		E 08 F 75	
	0.1	S/3			E 61 F 29
	0.01	S/10	A 260		



TABLE IX  
PLANT MATERIALS

Substance	Concentration	Media	Stimulating	Inert	Inhibitory toxic or lethal
Betaine (present in malva)	0.14% (10 mM)	Pl/3			L 63 F 6
Castilian malva (filtrate)	< 10 %	UF/10			A 0
Chlorophyll (Gruskin soln)	< 10 % < 100 %	UF/10		A 77	A 0
Chlorophyll (Rhystan powder 72% Chlorophyll)	0.2 % 0.1 % 0.05%	UF/3 Pl/3 Pl/3			EF 0 E 9 E 15 A 10

1 It may overcome the inhibitors present in dormant adult tissue. There is evidence that adult tissue cells are kept in a dormant state because of the inhibitory substances present in the tissues. Removal of inhibitors (by enzymatic digestion or other means) results in cell division. Agents that act in this manner may be looked upon as opposing the restraint of growth."

2 It may furnish nutrient or metabolic materials. Such materials may directly aid the cells in overcoming the tissue inhibitors (process 1), or they may increase the cell metabolism and indirectly serve to counteract the inhibitors or to promote mitosis.

3 It may act directly on the cell to promote mitosis. We have no evidence that any agent acts in this manner.

4 It may promote migration of cells either before or after division. In our experiments with adult aorta the migration of cells without mitosis is not mistaken for the growth of new cells.

Other observations tell us the length of the lag period before new cells are seen and also the rate and extent of subsequent cell division (with migration). In general a short lag period is followed by rapid growth while a long lag period precedes slow growth. This seems to hold true regardless of the nature of the stimulating agent.

In Table XI we have summarized the substances found to aid the growth of adult cells when added to media containing UF/3 or PI/3. We have not included those amino acids that stimulated in physiological solution but which failed to stimulate in UF/3. It will be noted that some substances may be stimulating to one type of cell and also be unfavorable for another type.

## VI SUBSTANCES RELATED TO WOUND HEALING (TABLE XII)

Numerous substances have been advocated by others (see reviews by Whipple 1940 and by Arey 1936) for the stimulation of wound healing. Tissue culture tests on a number of these materials are given in Table XII. It will be seen that less than half of these alleged stimulating agents showed any stimulation of adult tissues *in vitro*. Some were definitely toxic. Of those that stimulated none showed greater action than adult serum and none gave promise of practical usefulness in aiding the healing of wounds.

The substances in Table XII (as well as those in subsequent tables) were tested on experimental wounds by Dr. Edward L. Howes in the Department of Surgery. His findings were in general consistent with our results in tissue cultures—except for the fact that sterility is more of a problem in wounds than in tissue culture. An agent (such as serum

TABLE V  
INORGANIC COMPOUNDS

Substance	Concentration	Media	Stimulating	Inert	Inhibitory toxic or lethal
Aluminum chloride	0.0014% (0.1 mM) 0.003% (0.2 mM)	Pl/3 XG	F 180 A >200	E 121	
Barium chloride	0.005% (0.25 mM) 0.002% (0.1 mM)	Pl/3 Pl/3	F 236	E 125 E 104	
Cadmium chloride	0.004% (0.2 mM)	UF/10			A 0
Chromium chloride	<0.005% (0.2 mM)	UF/10		A 99	
Sodium chromate	0.005% (0.2 mM)	UF/10			A 0
Cupric chloride	0.003% (0.2 mM)	UF/10			A 0
Ferrous chloride	0.01% (0.5 mM) 0.01% (0.5 mM) 0.01% (0.5 mM) 0.002% (0.1 mM)	XG UF/3 Pl/3 Pl/3	A >200 E 167 E 150	E 73	
Ferric chloride	0.014% (0.5 mM) 0.005% (0.2 mM) 0.003% (0.1 mM) 0.001% (0.05 mM) 0.014% (0.5 mM) 0.014% (0.5 mM) 0.003% (0.1 mM)	XG XG XG XG UF/10 UF/3 UF/3	A >200 A 350 A 250 A 240 A 194	E 109 E 100	
Lead chloride	<0.1% (0.2 mM)	UF/3			A 57
Nickel chloride	0.005% (0.2 mM)	UF/10			A 0
Stannous chloride	0.005% (0.2 mM)	UF/10		A 113	

TABLE V (Continued)

Substance	Concentration	Media	Stimulating	Inert	Inhibitory toxic or lethal
Strontium chloride	0.013 % (0.5 mM)	N6	A 151		
	0.05 % (2 mM)	UF/10			A 28
	0.13 % (0.5 mM)	UF/10			A 35
Zinc chloride	0.003 % (0.2 mM)	UF/10			A 0
Metaphosphate in addition to 8.5 mM orthophosphate	0.08 % (10 mM)	PI/3			E 0
Metaphosphate without orthophosphate	0.05 % (8.5 mM) 0.05 % (8.5 mM)	PI/3 UF/3		E 127	E 38 A 45
"Calgon" (Sodium metaphos- phate in addition to ortho- phosphate)	0.01 %	PI/4		E 92 F >100	
	0.01 %	X6		A >100	

TABLE XI

SUMMARY OF SUBSTANCES FOUND TO BE STIMULATING TO ADULT TISSUE GROWTH<sup>a</sup>

Substance	Effect on the growth of		
	Aorta or skin fibroblasts	Skin epithelium	Thyroid epithelium
<b>Antibacterial substances</b>			
Sulfanilamide 0.005 and 0.05%	++		
Sulfadiazine 0.05 and 0.085%	+		
Sulfadiazine 0.05% (in Pl plus Z8) <sup>b</sup>		++	
Sulfamethazine 0.05%	+	++	++
Sulfathiazole 0.005 to 0.085%	+		
<b>Vitamins</b>			
Ascorbic acid 0.01% (in serum or UF/3)	+	+	
<b>Enzymes (preliminary digestion of tissues)</b>			
Papain 0.001 to 0.1%	+		
Trypsin 0.05 to 0.1%	++	Toxic	
<b>Hormones</b>			
Anterior pituitary 0.015 per ml (in S/4)	++		
Thyroid (fresh chicken) 4.0%	++	Inhibitory	
Thyroid (crude dried pig) <0.3%	+	Toxic	Inhibitory
<b>Other tissue extracts</b>			
Egg white (fresh or dried) 5% and 0.12%	++	++	
Embryo extract (chicken) 1/3 in UF/3	+		
Embryo extract (chicken) 1/3 in S/3	Inert		
Heart extract 10%	++	+	
Leucocyte extract 5% (in Ty)	++++ <sup>c</sup>		
Platelet extract 0.5%	+		
Red cell extract (laked) 0.1%	+		
Red cell extract (laked) 5.0%		++	
Chromatin 1.0%	++		
	(inconsistent)		

<sup>a</sup> Degree of growth stimulation is indicated as follows

Growth ratings

+ = up to 2 times that of the control medium

100-200

++ = between 2 and 3 times that of the control medium

200-300

+++ = between 3 and 4 times that of the control medium

300-400

++++ = between 4 and 5 times that of the control medium

400-500

+++++ = more than 5 times that of the control medium

&gt;500

Except where otherwise indicated the control medium was UF/3 or Pl/3

<sup>b</sup> However sulfadiazine 0.05% in Pl plus X6 was inhibitory<sup>c</sup> Activity compared with Ty or X6 solution control

TABLE VI (Continued)

Substance	Effect on the growth of		
	Aorta or skin fibroblasts	Skin epithelium	Thyroid epithelium
Plasma (and lymph) constituents			
Whole plasma lymph or serum	++++ <sup>c</sup>	++++ <sup>c</sup>	
Serum ultrafiltrate (A Factor)	++++ <sup>c</sup>		
Serum albumin 0.25%	Inert	+	
Fibrinogen 1/3	+	+	
Serum globulin 1/10	+	++	
Serum gamma globulin 0.25 to 0.5%	+	+	
Thrombin 0.25 to 0.5%	++	Inert	
Amino acids and digested proteins			
Casein (digested by pepsin) 1%	++++	++	
l Aspartic acid 7.5 to 15 mM	+	++	
l Asparagine 15 mM		++	
l Proline 7.5 to 15 mM	+	+	
Sulfur compounds			
Thiouracil 0.003%	+		+
Thiourea 0.003%		+	Inhibitory
Other organic compounds			
Aluminum monostearate <0.03%	+		
Aluminum tristearate <0.03%	+		
Inorganic compounds			
Aluminum chloride 0.003%	+		
Barium chloride 0.002%	+		
Ferrous chloride 0.01%	+	+	
Ferric chloride 0.01%	+		

ultrafiltrate or aspartic acid) which aids tissue cell growth can also aid bacterial proliferation

#### A. WATER SOLUBLE OINTMENT BASES (TABLE VIII)

Of the *water soluble bases* no inhibitory effect on the growth of adult fibroblasts was produced by the gels agar Irish moss methyl cellulose and gum tragacanth tested in a concentration of 1% or less. Irish moss inert for fibroblasts inhibited the growth of skin epithelial cells. The water soluble carbowaxes also had no inhibitory effect on the growth of fibroblasts when tested in a concentration of 1% but were less favorable for epithelial growth.

On the other hand pectin frequently recommended in the literature

TABLE VII  
TISSUE CULTURE TESTS ON SUBSTANCES REPORTED BY OTHERS TO BE STIMULATING  
TO WOUNDS<sup>a</sup>

Substance	Effect on Growth of Adult	
	Aorta or skin fibroblasts	Skin epithelium
Pectin	Toxic	
Glycerine	Toxic	
Triethanolamine	Toxic	Toxic
Cod liver oil fractions	Toxic	Toxic
Ascorbic acid	Stim +	Stim +
Yeast extract	Inert	Toxic
Trypsin (preliminary digestion)	Stim ++	Toxic
Adult serum (compared with 16 solution control)	Stim +++++	Stim +
Embryo extract	Stim +	
Adult tissue extracts		
Adrenal (whole)	Inert	
Heart	Stim ++	Stim +
Thyroid <sup>b</sup>	Stim ++	Inhibitory
Serum albumin	Inert	Stim +
Casein digest	Stim +++++	Stim ++
Glycine (impure)	Inhibitory	Inhibitory
Allantoin	Inert in 0.01% Toxic in 0.12%	
Urea	Inert in 0.05% Inhibitory in 0.09%	
Sodium oxalate	Inhibitory	

<sup>a</sup> The degree of growth stimulation is indicated as follows

	Growth ratings
+	100-200
++	200-300
+++	300-400
++++	>400

The control medium was UF/3 or PI/3 in each case except when serum and glutathione were tested

<sup>b</sup> When tested on thyroid epithelial cells thyroid extract was inhibitory thouracil was stimulating (+) and thiourea was inhibitory

TABLE XII (Continued)

Substance	Effect on Growth of Adult	
	Aorta or skin fibroblasts	Skin epithelium
Sulphydril compounds		
p Thioresol	Toxic	Toxic
Cysteine	Inhibitory	
Glutathione (not tested in UF)	(Stim ++ in AG)	
$\text{Na}_2\text{SO}_4$		Inhibitory
Thiouracil <sup>b</sup>	Stim +	
Thiourea <sup>b</sup>		Stim +
Castilian malva	Toxic	
Chlorophyll	Inert to Toxic	Toxic

for the treatment of wounds (Thompson 1938 Tompkins *et al* 1941) was completely toxic *in vitro*

A 10% concentration of glycerine reported as beneficial to wound healing by Kulkarni (1939) was found to be toxic for the growth of fibroblasts as was "glycerite" of starch. The addition of glycerine to inert tragacanth containing a noninhibitory concentration of sulfathiazole resulted in complete toxicity. Brush and Lam (1942) also report no consistent healing of guinea pig wounds with either pectin or glycerine although Robson and Wallace (1941) described a sulphonamide glycerine base as harmless in the treatment of burns.

## II WATER INSOLUBLE (GREASY) BASES (TABLE XIV)

The only *greasy bases* tested which were well tolerated *in vitro* were pure preparations of mineral oil and vaseline and a mixture of vaseline and lanolin ("Aquaphor"). These were tested by floating on the surface of a protective medium containing the tissue. Repeated heating of vaseline rendered it toxic. The importance of the quality of the preparation is evident from Table XIV which shows the variability of results obtained with different preparations.

Of a large number of bases, antiseptics and detergents tested on rat wounds by Baker (1944) petrolatum, motor oil and boric acid ointment were the only substances found not to cause necrosis. Hawking (1942) commented on the undesirable tissue reaction to oily preparations of the sulfa drugs used in treatment of wounds and recommended the use of saline solutions.



TABLE VIII  
WATER SOLUBLE OINTMENT BASES

Substance	Concentration (%)	Media	Stimulating	Inert	Inhibitory, toxic or lethal
Agar	0.5	UF/10		A >100	
Sodium alginate	1.0	UF/10			A 53
Aluminum hydroxide gel	<25	UF/5			A 0
	25	UF/10			A 0
	50	UF/10			A 0
Corn starch	1.0	UF/10			A 0
Ethyl cellulose	1.0	UF/3			A 0
Hydroxyethyl cellulose	1.0	UF/3		A >100	
Gelatine	5.0	UF/10			
Glycerine	1.0	UF/10			A 7
Glycerite of starch	1.0	UF/10			A <1
Gum acacia	2.0	UF/10			A 0
Irish moss	1.0	UF/3		A <100	A 13
Methyl cellulose	1.0	UF/3			F 37
					E 0
Pectin	1.0	UF/10		A 85 F 75 E 76	A 0
Polyvinyl alcohol	1.0	UF/10			A 0
Tragacanth	0.5	UF/10		A 83	
	1.0	UF/10		A >100	

TABLE VIII (Continued)

Substance	Concentration (%)	Media	Stimulating	Inert	Inhibitory toxic or lethal
Tragacanth glycerine base (0.5% sulfathiazole)	10	UF/10			A 0
Carbowax 1500	10	UF/3		F 71	E 37
Carbowax 4000	10	UF/3		A 76	E 10
Carbowax 8000	10	UF/3		F >100 E 83	
Navy SP EP ointment (containing beeswax and petrolatum)	S	UF/3			A 3
	S	Pl/3			E 68
	S	Pl/3			E 73

TABLE XV  
STEARATES AND GREASY BASES CONTAINING STEARATES

Substance	Concentration	Media	Stimulating	Inert	Inhibitory toxic or lethal
Hydrosorb (stearic acid grease)	S 100 %	UF/10		A 143	
Aluminum stearate grease (vaseline)	S 05 %	UF/10			E 56 F 14
Calcium stearate grease (mineral oil)	S 025 %	UF/3			EF 0
{ Calcium stearate grease 1/2 } { Almay mineral oil 1/2 }	S 100 %	UF/3			EF 0
Aluminum monostearate (Bristol Meyer)	P < 003 % ( 1 mM )	UF/3	A 193		
Aluminum tristearate (Bristol Meyer)	P 003 % ( 0.3 mM )	UF/3	A 195		
Aluminum tristearate (commercial)	009 % ( 1 mM )	UF/3			E 43 F 44
Barium stearate	P < 0035 % ( 0.5 mM )	UF/3		A 107	
Magnesium stearate	P < 003 % ( 0.5 mM )	UF/3		A 107	
Sodium stearate	P < 03 % ( 10 mM )	UF/3			L 14 F 25
Zinc stearate	< 03 % ( 0.5 mM )	UF/3		A 85	
Sodium 12 hydroxystearate	< 01 % ( 3.3 mM )	UF/3			LF 0
Glycerol monohydroxystearate	P 02 % ( 5 mM ) 10 % ( 25 mM )	UF/3 PI/3		E 79	LT 0

TABLE V (continued)

Substance		Concentration	Media	Stimulating	Inert	Inhibitory toxic or lethal
Diethylene glycol monostearate	S	1.0 %	(25 mM)	PI/3	E 130	E 28
		0.2 %	(5 mM)	UF/3		F 38
Nonacethylene glycol monostearate		< 0.1 %		UF/3		E 53
		< 0.2 %	(5 mM)	UF/3	E 30	E 14
Propylene glycol monostearate	P	1.0 %	(29 mM)	PI/3		F 25
Related compound						
Dodecaethylene glycol monolaurate		0.1 %		UF/3		EF 0

TABLE XVI  
 DETERGENTS EMULSIFIERS PENETRANTS SOLVENTS ETC

Substance	Concentration	Media	Stimulating	Inert	Inhibitory toxic, or lethal
Aerosol (sodium dialkyl sulfosuccinate)	1.0 %	UF/10			A 0
Duponol C (sodium lauryl sulfate)	1.0 %	UF/3			A 0
Ethyl silicate	0.1 % ( 5 mM)	UF/3			E 38 F 27 A 20
Lecithin (vegetable oil free)	1.0 % ( 50 mM)	UF/3			
1 2 Propanediol (propylene glycol)	0.008% ( 0.1 mM)	UF/3		A 110	E 60
2 Amino 2 methyl 1 3 propanediol	0.07 % ( 10 mM)	Pl/3		F 85	A 0
Polyethanediol (polyethylene glycol)	0.5 % ( 48 mM)	UF/9			L 60
	0.2 % ( 10 mM)	Pl/3			L 28
Polypropanediol (polypropylene glycol)	0.2 %	UF/3		A 119	
Sorbitol	1.0 % ( 52 mM)	UF/10		A 80	A 0
	10.0 % (524 mM)	UF/10			A 27
Sorbitan monooleate	1.0 % ( 22 mM)	UF/10			A 2
	3.3 % ( 74 mM)	UF/10			A 65
	0.1 % ( 2 mM)	UF/10			LF 0
Tergitol 08	1.0 %	UF/3			EF 0
Tergitol 7	1.0 %	UF/3			EF 0
Triethanolamine	0.2 % ( 15 mM)	Pl/3			LF 0
Triethanolamine with 0.09% sulfadiazine	0.2 % ( 15 mM)	XG and Ul/10			
Trichloroamine with 0.18% sulfadiazine	0.4 % ( 30 mM)	XG			A 0
No 260B 50 50 (Carbon and Carbide Co)	1.0 %	UF/3			A 0

(Reed and Orr 1942) Several hundred milligrams per 100 ml of sodium sulfathiazole was required to inhibit the migration of leucocytes *in vitro* (Herrell and Heilman 1943), and concentrations of 5 and 50 mg per 100 ml did not inhibit the growth of young guinea pig fibroblasts while concentrations of 100 mg per 100 ml inhibited growth but did not kill the cells (Reed *et al* 1942)

We have found (Table XVII) that concentrations of sulfanilamide and sulfathiazole as low as 5 mg per 100 ml will stimulate the growth of adult chicken erythrocyte fibroblasts in tissue culture and that a concentration of 50 mg per 100 ml of sulfanilamide, sulfadiazine, sulfamethazine or sulfathiazole usually had a stimulating effect on the growth of both adult fibroblasts and skin epithelial cells. Mafenide and sulfamerazine in the same concentration inhibited these cells. Sulfamethazine (50 mg per 100 ml) was found to be highly stimulating to the growth of adult chicken thyroid epithelium. The reaction of the tissues to different concentrations of sulfadiazine and sulfathiazole (5 to 85 mg per 100 ml) varied with the type of medium. A reduction in bicarbonate favored the stimulation of the growth of fibroblasts by the higher concentrations of sulfadiazine and sulfathiazole while a reduction in calcium and an increase in phosphate in the medium resulted in stimulation of skin epithelial cells by sulfadiazine. However sulfadiazine tended to inhibit these cells when applied in X6 solution which favors the growth of fibroblasts.

## 2 Miscellaneous Antibacterial Substances (Table XVIII)

In our experience the following commonly used antiseptics completely inhibited the growth of adult cells in tissue culture: boric acid 0.1%, hydrogen peroxide 0.01 to 1.0%, iodine <0.05%, phenol 0.5%, "Zephiran" 0.02%, zinc peroxide <0.01%. Baker (1944) found boric acid ointment to be harmless in the treatment of rat wounds but stated that hydrogen peroxide caused necrosis of the muscle. Zinc peroxide has been recommended as nonirritating (Holmes 1942) and growth stimulating (Connell 1940) in the treatment of wounds. According to Herrell and Heilman (1943) "Zephiran is hemolytic in low concentrations and although inferior to penicillin and sodium sulfathiazole compares favorably with gramicidin in regard to toxicity for leucocytes in tissue cultures. We have found gramicidin to be relatively inert in tissue cultures of adult fibroblasts and skin epithelial cells.

In addition to completely inhibiting growth *p*-chlorophenol, iodine, phenol, proflavine and propamidine delayed plasma clotting from 1 to 4 days. *p*-Chlorophenol recommended for use with penicillin by Melaney *et al* (1946) because of its activity against gram negative organ-

TABLE XVII  
 ANTIBACTERIAL SUBSTANCES A SULFA DRUGS

Substance	Concentration	Media	Stimulating	Inert	Inhibitory toxic or lethal
Marfanil (4 amino 2 methyl benzene sulfonamide)	0.05 % ( 2.5 mM)	UF/3			A 48
	0.1 % ( 5 mM)	UF/3			A 0
	0.1 % ( 5 mM)	PI/3			E 10
	0.5 % ( 2.5 mM)	PI/3			F 0
					F 44
Sulfanilamide	0.01 % ( 0.5 mM)	PI/3			E 24
	0.005% ( 0.3 mM)	UF/10	A 140		E 40
	0.05 % ( 2.9 mM)	UF/10	A 70		
	0.05 % (2.9 mM)	UF/10			
	0.05 % ( 2.9 mM)	X6	A 250		A 3
Sulfadiazine	0.085% ( 3.4 mM)	D UF/10	A 175		
	0.009% ( 0.3 mM)	D UF/10	A 150		
	0.05 % ( 2 mM)	UF/10	A 131		
	0.085% ( 3.4 mM)	UF/10			
	0.085% ( 3.4 mM)	X6		A 102	
	0.05 % ( 2.0 mM)	X6			A 56
	0.005% ( 0.2 mM)	X6			A 53
	0.05 % ( 2.0 mM)	PI/3 (X6)			A 71
	0.05 % ( 2.0 mM)	PI/3 (Z8)	E 260		E 52
	0.05 % ( 2.0 mM)	UF/3			A 68
Sulfamerazine	0.05 % ( 2.0 mM)				

TABLE XVII (Continued)

Substance	Concentration	Media	Stimulating	Inert	Inhibitory toxic or lethal
Sulfamethazine	0.05 % ( 2.0 mM)	UF/10	A 143		
	0.05 % ( 2.0 mM)	Pl/3 (X6)	E 155		
	0.05 % ( 2.0 mM)	Pl/3 (Z8)	E 210 Thy 280		
Sulfasuxidine	0.10 % ( 2.8 mM)	X6	A 175		
	0.10 % ( 2.8 mM)	UF/10		A 73	
Sulfathiazole	0.085% ( 3.3 mM)	D UF/10		A 130	
	0.05 % ( 2.9 mM)	UF/10	A 174		
	0.085% ( 3.3 mM)	UF/10		A 90	
	0.005% ( 0.2 mM)	UF/10	A 210		
	0.005% ( 0.2 mM)	X6		A 22	
	0.085% ( 3.3 mM)	X6		A 43	



TABLE XVIII  
 ANTIBACTERIAL SUBSTANCES B MISCELLANEOUS

Substance	Concentration	Media	Inert	Inhibitory toxic or lethal
9 Amino acridine HCl	0.0005 % ( 0.03 mM)	UF/3		A 0
Benzyl alcohol	0.1 % ( 10 mM)	Pl/3		E 8 F 0
Boric acid	0.09 % ( 15 mM)	UF/10		A 15
Carboxymethoxyamine	0.09 % ( 10 mM)	UF/3	A 90	EF 0
	0.09 % ( 10 mM)	Pl/3	F 70	E <1
	0.045 % ( 5.0 mM)	Pl/3	F 100	E 16
Acetoxime of carboxymethoxyamine	0.13 % ( 10 mM)	UF/3	A 111	E 67 F 65 F 60 E 10
	0.13 % ( 10 mM)	Pl/3		
Cyclohexanoneoxime of carboxy methoxyamine	0.17 % ( 10 mM)	UF/3	A 118	E 17 F 5
	0.17 % ( 10 mM)	Pl/3	E 125 F 108	
	0.02 % ( 1.0 mM)	Pl/3	E >100 F >100	
p Chlorophenol	0.1 % ( 8.0 mM)	Pl/3 <sup>a</sup>		EF 0
	0.01 % ( 0.8 mM)	Pl/3		EF 0
	0.001 % ( 0.08 mM)	UF/3		A 0

<sup>a</sup> These solutions delayed the clotting of the plasma

TABLE XVIII (Continued)

Substance	Concentration	Media	Inert	Inhibitory toxic or lethal
Gramicidin	P <0.1 %	UF/10		A 44
	P <0.01 %	UF/10		A 53
	0.005 %	UF/10	A 119	
	0.001 %	UF/10	A 120	
	P <0.1 %	UF/3	E 130	E 39
	P <0.1 %	PI/3	F 103	
		PI/3	E >100	
	P <0.01 %		F 75	
Hydrogen peroxide ( Superoxid )	10 % ( 300 mM )	UF/10		A 0
	0.1 % ( 30 mM )	UF/10		A 0
	0.01 % ( 30 mM )	UF/10		A 0
8 Hydroxyquinoline	P <0.1 % ( 70 mM )	PI/3		EF 0
	P <0.1 % ( 70 mM )	UF/3		A 0
	0.01 % ( 0.7 mM )	PI/3		EF 0
	0.01 % ( 0.7 mM )	UF/3		A 0
Iodine	P <0.05 % ( 20 mM )	PI/3*		EF 0
Isonitellin	0.7 % ( 500 u/ml )	UF/10		A 0
	0.07 % ( 50 u/ml )	UF/10		A 0
	0.007 % ( 5 u/ml )	UF/10		A 5
	0.0015 % ( 1 u/ml )	UF/10		A 31
Barium penicillin	0.07 % ( 100 u/ml )	PI/3		EF 0
	0.03 % ( 50 u/ml )	PI/3		EF 0
	0.007 % ( 10 u/ml )	PI/3		EF 0

Substance	Concentration	Media	Inert	Inhibitory toxic or lethal
Calcium penicillin	0.03 % (100 u/ml)	UF/3	A 90	
	0.003 % (10 u/ml)	UF/3	A >120	
	0.003 % (10 u/ml)	PI/3	E 111	
	0.02 % (100 u/ml)	PI/3		A 61
Sodium penicillin	0.002 % (10 u/ml)	UF/3	A >120	
	0.02 % (100 u/ml)	UI/3		A 28
	0.02 % (100 u/ml)	PI/3		E 57
	0.002 % (10 u/ml)	PI/3		E 69
Phenol	0.5 % (50 mM)	PI/3 <sup>a</sup>		EF 0
Proflavine 2 HCl	0.07 % (2.3 mM)	PI/3 <sup>a</sup>		EF 0
	0.025 % (0.9 mM)	PI/3 <sup>a</sup>		EF 0
	0.005 % (0.18 mM)	PI/3 <sup>a</sup>		EF 0
	0.07 % (1.7 mM)	PI/3 <sup>a</sup>		EF 0
Propamidine 2 HCl	0.025 % (0.7 mM)	PI/3 <sup>a</sup>		EF 0
	0.005 % (0.1 mM)	PI/3 <sup>a</sup>		E 0
				E 0
				Γ 20
Protoanemonin	0.0004 %	PI/3		LF 0
	0.00002 %	PI/3 (X6)		E 43
				F 32
	0.0001 %	PI/3 (X6)		E 0
8 Quinolyl benzoate	0.00002 %	PI/3 (Z16)	F 88	L 71
	0.0001 %	PI/3 (Z16)		E 16
				F 18
	0.01 % (0.4 mM)	UF/3		A 0
	0.001 % (0.04 mM)	UI/3		A 0

TABLE XVIII (Continued)

Substance	Concentration	Media	Inert	Inhibitory toxic or lethal
Streptomycin HCl	0.004 % ( 20 mM ) 0.04 % ( 200 u /ml )	UF/10 UF/10	A 105	A 17
Streptothricin HCl	0.02 % ( 20 u /ml ) 0.2 % ( 200 u /ml )	UF/10 UF/10		A 37 A 1
Sulfur preparation S968A (Weld)	0.00004 % 0.000001 %	UF/3 UF/3	A 97 A 109	
p Toluquinone	0.1 % ( 80 mM ) 0.1 % ( 80 mM ) 0.01 % ( 0.8 mM ) 0.01 % ( 0.8 mM ) 0.001 % ( 0.08 mM )	UF/3 Pl/3 <sup>a</sup> UF/3 Pl/3 UF/3		A 0 EF 0 EF 0 EF 0 A 0
Tyrosine (Alcohol 0.5%)	P < 0.05 %	UF/3		A 60
Vioform (Iodochlorhydroxyquinoline)	0.0005 % ( 0.015 mM ) 0.001 % ( 0.03 mM ) 0.0001 % ( 0.003 mM )	UF/3 UF/3 Pl/3	A 80	EF 0 EF 0 E 62
Vioform in 0.1% propylene glycol	0.0005 % ( 0.015 mM )	UF/3		A 0
Zephiran (Benzyltrialkyl ammonium chloride)	0.02 % 0.01 %	UF/10 UF/10		A 0 A 0
Zinc peroxide	P < 0.01 % ( 1.0 mM )	UF/3		A 0



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## CHAPTER 8

# Food Energy and the Metabolism of Nitrogen<sup>1</sup>

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## I INTRODUCTION

The demonstration by Magendie in 1816 of the unlike nutritive value of the three major groups of foodstuffs (Magendie 1816) spearheaded in the latter half of the nineteenth century an era of research activity relating to the influence of the nonprotein calories of the diet on the course of protein metabolism. The literature is dotted with the names of the great investigators of the period—Bischoff Rubner Voit Lusk Atwater and many others. It is not surprising that the work of men of this caliber culminated in concepts that have been more or less generally accepted even up to recent times. Many have the familiarity of textbook statements. For example we may read: Protein is metabolized less economically as a sole dietary constituent than it is in the presence of carbohydrate or fat although both carbohydrate and fat have protein sparing characteristics carbohydrate is the more efficient of the two the nonprotein foodstuffs spare protein by virtue of their caloric prop-

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erties, and the total energy value of the diet determines the level at which the protein metabolism proceeds

A bringing together, however, of the old and the new literature suggests that many factors not appreciated by the early workers in the field influence the effect nonprotein calories may exert on protein metabolism (Munro, 1951). As a result, the picture has been enlarged, many of the seeming contradictions cleared, and inconsistencies with the isodynamic law explained. But at the same time, new problems and new questions have arisen.

The better understanding that has come in recent years of the role of calories in the regulation of nitrogen metabolism has its roots in our concept of the living cell as a steady or dynamic system rather than as one of equilibrium (Schoenheimer *et al.*, 1939). And as such it is an open system—materials enter and leave and the state is determined by kinetic factors—a situation quite in contrast to the closed state of a thermodynamic equilibrium (San Pietro and Rittenberg, 1953). This dynamic state has been viewed as an ebb and flow of nitrogenous constituents from one tissue to another (Whipple, 1948). In this system, both dietary and tissue proteins contribute amino acids to a metabolic pool which supplies in turn all of the amino acids needed for both anabolic and catabolic processes. Actually this pool represents the summation of protein metabolism in many centers integrated into the dynamic state of the body as a whole (Allison, 1957).

In addition, certain tissue proteins notably those of the liver, in testines and plasma are more labile than others and have, in addition, the capacity of holding in reserve varying amounts of protein. In this fluidity of the protein metabolism and its immediate response to physiological dietary and environmental variables is found the body's adaptive abilities and its power to shift the course and the intensity of metabolic processes in response to normal, subnormal, and surfeit conditions of various origins.

Within this conceptual framework it therefore becomes imperative to define observations relating to the influence of nonprotein calories on the protein metabolism in terms of the experimental conditions imposed—the age of the test animal, its physiological condition, the size of its stores of protein, fat, and glycogen within its body, the relative adequacy of the diet offered, and the scheme of the dietary regime to which the test animal is subjected, including pre-feeding techniques, duration of the experimental period, and length of intervals between dietary phases of the experiment.

## II INFLUENCE OF TOTAL ENERGY VALUE OF DIETS ON PROTEIN METABOLISM

Recent studies (Rosenthal and Allison 1951 Calloway and Spector 1955 Forbes and Yohe 1955 Schwimmer and McGavack, 1948 Leverton *et al* 1951) emphasize the importance of balance between the energy producing and protein components of the diet in protecting the integrity of body tissue under normal conditions. Labile protein in body reserves through catabolic processes provides energy as well as amino acids in support of activities both anabolic and catabolic in the metabolic pool. In general a restriction in the energy value of the diet is associated with an increased catabolism of labile protein in the effort of the animal to correct the caloric deficiency in the metabolic pool.

Conversely increments in food energy value permit improved utilization of food nitrogen for synthesis and conservation of body protein but there appears to be an optimum caloric intake associated with maximum retention at various levels of dietary protein (Calloway and Spector 1954).

Also an optimum level of calories seems to exist for the maintenance of an efficient balance between fat stores and lean body mass (Allison and Wannemacher 1957). For example puppies gained essentially the same body weight per gram of nitrogen ingested on diets containing either egg protein or wheat gluten as a source of protein. The puppies fed wheat gluten however retained only 0.08 gm of nitrogen per gram of nitrogen intake whereas those given egg protein retained 0.5 gm per gram of nitrogen consumed. The gluten fed puppies were obese and inactive the egg protein fed animals lean and active.

Whether catabolism or anabolism predominates in the body following caloric restriction depends on the degree to which the energy value of the diet has been reduced, the quantity and nutritive value of the dietary protein, the physiological state of the animal in respect to body reserves of protein and fat, the age of the animal and in some instances the dietary sources of energy. It is not difficult to see therefore that no one metabolic picture defines the response of an experimental animal to a reduction of food energy. However the many and complicated metabolic adaptations that an animal may make under various conditions of caloric restriction are becoming clear as evidence accumulates in various laboratories. Emerging concepts have been summarized by Allison (1958).

### A EFFECT OF PROTEIN CONTAINING DIETS WHEN FED TO ADULT SUBJECTS

When food ingested is of constant protein value and body stores of protein are filled reduction of food energy below the amount needed for maximum protein anabolism results first in a state marked concomitantly

by an increased catabolism of body protein and continued efficiency of utilization of food protein for anabolism—is shown by negative nitrogen balance and no alteration in the value of the nitrogen balance index of the dietary protein (Allison *et al*, 1946) Tissue synthesis reflecting such utilization may occur even with extreme reduction of caloric value when the protein content of the diet is low (Allison *et al*, 1946 Swanson, 1951, Benditt *et al*, 1948 Cox *et al*, 1953) But the body will burn any additional protein added to the diet as fuel, if caloric restriction is continued (Allison *et al*, 1946, Swanson 1951)

## II EFFECT OF VARYING THE PROTEIN STORES OF THE ANIMAL

That there are alternative pathways in metabolism is illustrated by differences in the way in which animals with full and depleted body stores of protein respond to a caloric deficiency

Caloric requirements are maximum when protein stores are filled With restriction of food energy, in animal possessing such stores will dip deeply into its reserves catabolize them for energy, eliminate in the urine nitrogen arising in the process, and thereby slip into nitrogen deficit even though there may be simultaneous anabolism of food protein (Rosenthal and Allison, 1951)

As body stores of labile protein are reduced following restrictions in the energy value of the food provided the caloric requirement seems to decrease permitting more effective utilization of amino acids for anabolic processes (Rosenthal and Allison 1951 1956) Thus with continued limitation of calories some adaptation seems to occur, as shown by drifts toward equilibrium and even in some instances, attainment of a positive nitrogen balance

Indeed the first response of a protein depleted animal to protein feeding and a deficiency of calories may be positive nitrogen balance

However such adaptations as occur are but transitory Eventually, in its search for sources of energy, the organism is forced to burn not only food but tissue protein for fuel as shown by an alteration in the nitrogen balance index and a markedly increased negative nitrogen balance The increased catabolism leads to irreversible tissue changes, exhaustion and finally death of the animal

Resistance to the final damaging response in which both food and body nitrogen are used for energy purposes is much greater under the stress of reduced calories when initial body reserves are full rather than depleted

The filling of reserves in the protein depleted animal presents another interesting example of protein calorie relationships Allison and Wanner (1957) present clear cut evidence showing that the filling of the

protein reserves must be described in terms of three dimensions namely (1) the nutritive value of the dietary protein (2) the nitrogen intake and (3) the caloric intake"

### C EFFECT OF PROTEIN FREE DIETS WHEN FED TO ADULT SUBJECTS

The catabolism that follows the feeding of a protein free diet represents after the initial adjustment an endeavor on the part of the organism to secure nitrogenous metabolites essential for the maintenance of life (Brush *et al* 1947). Labile stores of nitrogen are first drawn upon and then upon their exhaustion the more stable masses of body protein.

When protein free diets are administered to adult rats nitrogen balance does not serve as a sensitive indicator of the energy content of the diet. Altering the caloric consumption of adult rats in a range close to the maintenance requirement has very little influence on the quantity of nitrogen excreted in the urine (Mitchell 1924 Treichler and Mitchell 1941). Only with marked reductions in caloric value of the diet are there increments in nitrogen output.

With standardized protein depleted rats a catabolism greater than that characteristic when the full caloric protein free diet is fed does not occur until the calories have been reduced by more than one half (Willman *et al* 1947). Human subjects consuming protein free diets respond similarly to decreases in the energy value of the food (Johnson *et al* 1947 Cilloway and Spector 1954).

The dog appears to have the capacity to react favorably to super maintenance levels of energy producing foods when subsisting on a protein free ration (Allison and Anderson 1945).

### D EFFECT OF VARYING THE SOURCES OF FOOD CALORIES

The relative efficiencies of carbohydrate and fat in the sparing effect of nonprotein calories on the utilization of protein and on the course of nitrogen metabolism have been studied under a variety of experimental conditions over the years (Munro 1951 Maignon 1933). The adaptive responses and variations in metabolic mechanisms already discussed as functions of the energy and protein values of the diet explain in part at least divergence and confusion in data reported in the literature concerning the respective values of carbohydrate and fats in protecting the protein metabolism.

The preponderance of evidence to date suggests that in general the nitrogen conserving effects of carbohydrate and fat calories are equivalent in normal adult animals when diets contain protein providing measurements of retention are made for sufficiently long periods of time. The increase in urinary nitrogen that has been observed when fat is ex-

changed isocalorically for carbohydrate appears to be a transitory phenomenon (Thomson and Munro 1955)

The equivalence in the protein sparing effects of carbohydrate and fat is apparent in rats at various levels of caloric intake (Calloway and Spector, 1955, French *et al*, 1948) and in dogs receiving 50% or more of their food energy requirements (Allison *et al*, 1946 Rosenthal 1952)

In the case of the dog neither the utilization of the dietary protein (nitrogen balance index) nor the rate of catabolism seems to be altered with changes in the fat content of the diet when its caloric value is optimal (Rosenthal, 1952) However, when the intake of energy food meets only 25% of the requirement, both the utilization of dietary protein and the nitrogen balance are affected unfavorably if the diet is rich in fat—a response that is offset when dietary protein is increased

Some of the contradictions in the literature have been cleared by observations that separation in time of eating the protein and carbohydrate components of the food results in a deterioration of the nitrogen balance (Cuthbertson and Munro 1939 Geiger *et al*, 1950 Munro 1949) Thomson and Munro write that the initial accelerating effect on nitrogen metabolism of the isocaloric substitution of fat for carbohydrate may be due essentially to removal of carbohydrate from the protein containing meal and not to an adverse effect of feeding fat with protein (Thomson and Munro, 1955)

These observations together with others on the influence of surfeit isodynamic amounts of either fat or protein superimposed on adequate basal diets, led Munro and Wikramanayake (1954) to conclude that utilization of protein is affected in two ways by the carbohydrate present in the diet The first effect is specific and is one not observed with lipids A close proximity in time of eating carbohydrate and protein is necessary if retention of nitrogen is to be influenced favorably The second effect is nonspecific carbohydrate and fat act interchangeably as energy sources in sparing protein and to exert this sparing action do not need to be taken along with dietary protein

It has been more or less accepted from limited evidence reported in the early literature that when rations are deficient in protein nitrogen metabolism proceeds with less detriment to the animal when high carbohydrate rather than high fat diets are ingested (Landergrén 1903 Cathcart 1922) However diets were inadequate in many respects and it was not always recognized that response is a function of the total energy value of the diet and physiological reserves of fat glycogen and labile protein

More recent evidence indicates that fat possesses under specific experimental conditions nutritive properties over and beyond the furnish

ing of energy that are not exhibited by carbohydrate (Swanson 1951 Willman *et al* 1947 Swanson *et al* 1959)

The response of protein depleted rats to the simultaneous elimination of dietary protein and restriction of food calories as observed in the Iowa State College laboratories is providing evidence of the inextricable interweaving of the nitrogen and energy metabolism and an interesting opportunity to study alterations in metabolic pathways which may occur when stress conditions are imposed

### III INFLUENCE OF NONPROTEIN CALORIES ON ENDOGENOUS NITROGEN METABOLISM

#### A EXPERIMENTAL CONDITIONS

Adult male rats of Wistar strain with body reserves of labile protein reduced under standard conditions were the reactors in the Iowa State College experiments to the triple stress of previous protein depletion continued feeding of a diet devoid of protein and insufficient food energy

Two basic diets were used—one containing 20% of fat with the lipid constituent in most cases providing about 36% of the total calories the other nearly fat free Although the caloric densities of the two rations differed quantities of food consumed equalized the caloric values of the food ingested each day when the two diets were fed *ad libitum* (Table

TABLE I  
COMPOSITION OF LOW NITROGEN BASAL DIETS

Dietary components	High fat diet (grams)	Low fat diet (grams)
Dextrin	73	93
Lard	10	0
Butterfat	10	0
Osborne & Mendel salts	4	4
Sodium chloride	1	1
Ruffex	2	2
Calories per gram	4.8	3.8
Average calories provided per day <i>ad libitum</i> ingestion	49 (18 rats)	49 (36 rats)

1) These basal diets were supplemented daily with all of the known vitamins including choline and with 50 mg of cottonseed oil as a source of essential fatty acids

Various sources of fat have supplied the fat moiety over the course of the experiment We have used a mixture containing butterfat and lard in equal proportions Wesson oil butterfat alone lard alone, pure cottonseed oil of known origin and composition a low pressure distillation

fraction of cottonseed oil, a low pressure distillation residue of cotton seed oil and three preparations of a partially hydrogenated cottonseed oil. A mixture of butterfat and lard served as the source of fat in the first series of experiments.

The rats were maintained on the two protein free diets offered *ad libitum* until stabilization of nitrogen excretion showed adjustment to lack of dietary protein (Swanson 1951). It was believed that steady excretion of nitrogen signified a reduction not only in the size of body stores of protein but in their catabolic activity. Excretion of urinary nitrogen was fairly constant from the nineteenth through the thirty fifth day of protein restriction. *Metabolism experiments involving caloric reduction* were conducted in this interval.

The first nitrogen balance test, initiated on the nineteenth day, covered a period of 5 days the animals having full access to one of the nitrogen low rations. This experimental interval is designated as Period I in the tables in which data are presented.

In many experiments the animals in both the high fat and low fat groups were divided into two groups at the end of Period I. The first group received daily a quantity of food that represented the average amount consumed *ad libitum* per day during the first balance test i.e. adequate calories. The second group received a quantity of food reduced to some fraction of the amount consumed in Period I i.e. restricted calories. Each group was maintained on the designated dietary regime for 4 days—an interval long enough to permit adjustment of the animals on reduced caloric intakes to the new dietary manipulation. Then nitrogen balances were again determined for either one or two intervals, each 5 days long (Periods II and III).

The plan permitted comparisons of the performance of a single group of rats receiving full or restricted calories (longitudinal) as well as comparisons between different groups fed the two diets in exactly the same interval of the experiment (cross sectional). Except in survival studies all experimental groups throughout the entire investigation contained at least 6 rats.

Due to the uniformity of the nitrogenous composition of the fecal excretions under the experimental conditions imposed urinary nitrogen excretion only was used as the index of response to the test diets in some of the experiments.

## B. DIETARY FAT AND NITROGEN CATABOLISM

That a mixture of butterfat and lard consistently exerts a specific sparing effect on the nitrogen catabolism of a standard protein depleted rat is clear from data presented in Table II covering seven years of study.

TABLE II  
MEAN URINARY NITROGEN AND MEAN NITROGEN BALANCE OF GROUPS OF RATS PARTIALLY DEPLETED OF BODY RESERVES  
OF PROTEIN WHEN LOW FAT AND HIGH FAT RAT RATIONS ARE FED<sup>a</sup>

Group	Source of data	Date of experiment	Low fat ration			20% fat ration		
			Period I	Period II	Increase in catabolism Period I to Period II	Period I	Period II	Increase in catabolism Period I to Period II
Urinary nitrogen (milligrams per 5 days)								
A	All rats living and non moribund at end of experiment (60 rats)	1946	190	679	489	236	408	170
		1947	178	595	407	207	390	183
		1947	220	695	465	207	398	191
		1950	148	695	547	173	313	140
		1952	149	478	329	191	114	253
		Mean	177	624	447	203	399	185
B	Rats meeting specific physiological criteria	1950	151	395	244	173	313	140
		1952	151	386	235	193	377	184
		Mean	151	390	239	183	345	162
Nitrogen balances (milligrams per 5 days)								
A	All rats living and non moribund at end of experiment (44 rats)	1946	-310	-724	414	-231	-418	187
		1947	-225	-725	500	-235	-412	177
		1947	-285	-635	350	-303	-417	114
		1950	-246	-739	393	-239	-343	104
		Mean	-266	-706	440	-252	-397	145
B	Rats meeting specific physiological criteria	1950	-241	-434	193	-239	-343	104

<sup>a</sup> Adequate calories in Period I and restricted calories in Period II and with dietary fat provided by a 1:1 mixture of butterfat and lard



Restriction of the calories provided by either diet to 25% of the required number is accompanied by acceleration of catabolic activities as shown by comparison of excretions of urinary nitrogen and nitrogen balances in Periods I and II (Group A). But, at this caloric level incorporation of fat in the diet inhibits the extent of the acceleration. For example the increase in the negativity of the nitrogen balance is 440 mg in the 5 day metabolism period for the animals receiving the low fat diet and only 145 mg for the rats maintained on the fat containing diet.

In this connection, other data show that nitrogen balances vary only in the range of  $\pm 10$  to 15 mg in Periods I and II when the experimental animals are continued on the protein deficient diet without limitation of caloric consumption in Period II.

However, the possibility that the nitrogen catabolism reflects the physical condition of animals subjected to these harsh experimental conditions rather than the diet can not be dismissed. Some rats exhibited severe infections of the lungs at necropsy. Qualitative appraisal suggested that size of fat depots varied. Occasionally animals lost weight precipitously during the period of caloric restriction.

It seemed important therefore, to restrict comparisons to the healthier individuals in each group. A plan was developed for scoring the physical condition of the animals.

Data pertaining to any rat were withdrawn if two or more of the following conditions prevailed: moderate to severe lung infection, marked loss in weight or absence of body fat (peritoneal, subcutaneous and intramuscular). Data describing the remaining animals are summarized in Group B, Table II.

Comparisons of Groups A and B show that rats receiving the restricted protein free diet low in fat in Period II are affected particularly by the withdrawal procedure. However the distinction between the fat fed group and those relying on carbohydrate for their food energy is not lost.

Whether this screening of data is justified in the interpretation of results is a moot question. It should be noted that certain of the symptoms on which the screening was based represent advanced symptoms of protein deficiency and that the screening especially affected the rats fed the diet containing carbohydrate only. The tearing down of body tissue that occurs in these rats may be a true reflection of a difference in the nutritive value of the carbohydrate and fat diets as fed in this investigation and may represent inability of individual protein depleted rats to cope with the added insult of omission of dietary fat. Data also show that mortality of the rats fed the high carbohydrate diet is higher even on full calories than it is among rats receiving the fat diet (Swanson 1951). We continue to note in recent experiments that the presence of

fat in the ration in all instances delays death of the animal beyond the experimental period

### C LEVEL OF CALORIC INTAKE

There is a level of caloric intake associated with the sparing effect exerted by dietary fat on the endogenous metabolism when a mixture of lard and butterfat is used in the ration (Fig 1) The nitrogen catabolism

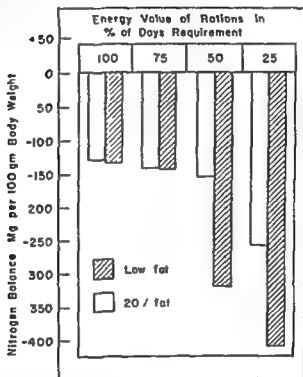


FIG 1 Nitrogen balances when protein free rations of varying fat content are fed to protein depleted rats at different caloric levels

is not accelerated greatly when the caloric value of the day's ration is reduced to one half that of the full *ad libitum* intake providing the ration contains fat. Such is not the case when the animal must rely on carbohydrate as its source of energy. A 50% reduction in the number of calories furnished by the diet results in a marked increase in catabolic processes which is accentuated upon further reduction in the energy value of the food intake (Willman *et al* 1947 Swanson *et al* 1959)

### D QUANTITY OF FAT IN THE DIET

Also the quantity of fat in the diet is important in the protective action exerted against the disintegration of body tissue that is induced by severe caloric restriction in protein depleted rats given rations low in

nitrogen (Willman *et al*, 1947 Swanson *et al*, 1959) Seemingly, the diet must carry at least 15% of fat if it is to exert nitrogen sparing activity against severe restriction of calories (Fig 2) In this case, a 1:1 mixture of lard and butterfat represents the lipid component of the ration

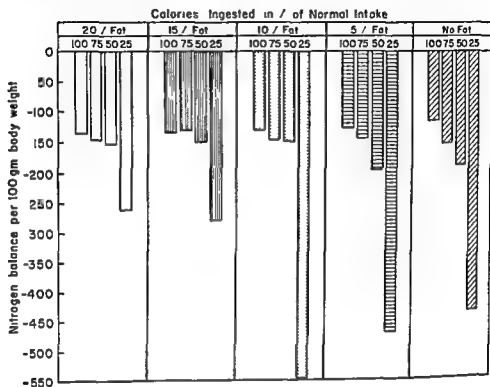


FIG 2 Nitrogen balances following the feeding of protein free rations of varying fat content and of graded caloric value to protein depleted rats

### E PATTERNS OF CATABOLISM AND SURVIVAL

The shifting nature (Rosenthal and Allison, 1956) of the nitrogen metabolism in rats possessing reduced stores of body protein when low calorie protein deficient diets are fed over a prolonged period is apparent from the data depicted in Fig 3 (Fox *et al*, 1959a Fox 1954) However fat in the diet alters the general metabolic response; animals receiving this nutrient seemingly have a capacity to adjust both the caloric and protein economies in a way that delays the eventual outcome death

The inclusion of fat in the ration (Wesson oil) maintains the catabolism at a fairly low plane over the entire remaining life span of the rat. Of interest is the adaptation indicated by the marked reduction in the overall catabolic activity during the last 10 days of life. When fat is absent from the diet, this final adaptation is not accomplished. Indeed

early nitrogen losses are insurmountable and death ensues relatively quickly.

Apparently a total loss in body nitrogen in the neighborhood of 3500 mg can be tolerated by the protein depleted animal before death intervenes after caloric restriction (Table III). However, it is the rate at which this loss occurs that determines length of life and in this case we note the favorable influence of dietary fat.

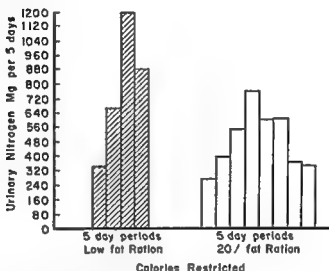


FIG. 3 Mean quantities of nitrogen excreted in urine in successive 5 day intervals by groups of protein depleted rats when the energy value of a protein deficient diet is restricted to 25% of the requirement (4 rats per group)

On the other hand the feeding of a protein free ration at full caloric value reverses the situation (Fig. 4) and the sparing effect of dietary fat on the endogenous nitrogen metabolism is lost (Fox *et al.* 1959a). The quantity of nitrogen excreted in the urine is greater for a fairly long interval of time (about 70 days) when fat is included in the diet than when it is omitted. At the end of this period however catabolism of the fat fed rats steadies and resembles that of the animals given the carbohydrate diet.

The greater catabolic activity occurring in the initial phases of the test in depleted fat fed rats receiving ample calories but deprived of protein is reflected in time of survival. The records in Fig. 4 are of special interest in that they show the extent to which the metabolism both energy and protein is reduced with the passing of time as the animal attempts to maintain life. It is remarkable that an animal with limited body reserves of protein can survive for as long as 200 days when protein deficiency is imposed. These data also point up that due

TABLE III  
SURVIVAL TIME CUMULATED LOSSES IN BODY NITROGEN AND LOSSES IN BODY WEIGHT OF RATS FED VARIOUS DIETS  
FROM TIME THAT CALORIC RESTRICTION WAS INITIATED UNTIL DEATH<sup>a</sup>

Diet	Number of rats	Survival on experimental regime (days)	Losses in body nitrogen (mg)		Losses in body weight (gm)	
			Total	Per day	Total	Per day
Low fat 12 Cal/day	4	24	3049	127	114	48
	5	27	2904	108	122	45
	6	31	3580	115	142	46
	8	29	3401	117	135	46
	Mean	28	3233	115	128	46
20% fat 12 Cal/day	35	42	3972	95	127	30
	36	46	4038	88	141	30
	37	39	3573	92	128	33
	38	46	3571	78	140	30
	Mean	43	3789	88	134	31

<sup>a</sup> Dietary fat provided by Wesson oil

to the changing nature of the nitrogen metabolism with the progression of time and statements regarding the relative nitrogen sparing properties of carbohydrates and fats should take time intervals in experimental history into consideration

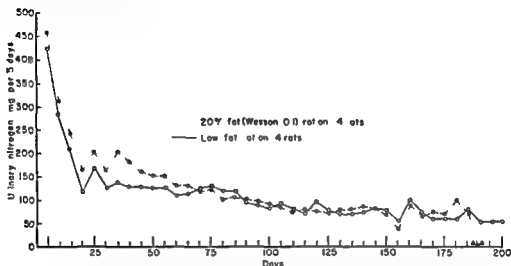


FIG. 4. Mean quantities of nitrogen excreted in urine in successive 5 day periods by groups of rats fed different protein free rations *ad libitum*. The symbol \* denotes that rats began to die at this point. From the 155th day values are for surviving rats. \*\* denotes that all rats receiving the high fat diet are dead.

#### F BODY SPARING PROPERTIES OF VARIOUS FATS

Some of the divergence of opinion regarding the relative protein sparing properties of carbohydrate and fat may be traced to the kind of fat used in the formulation of the ration. Fats as a group do not constitute a nutritional entity. Sources of the fat components of an experimental diet need to be defined as explicitly as sources of protein. Failure of investigators to recognize this fact has clouded many issues particularly since specific fats obtainable in local markets vary in nature and composition from time to time.

Early tests in this laboratory (Fox *et al* 1952) suggested that although certain irregularities in response were observed fats derived from various sources may vary in their protein sparing effect on the endogenous nitrogen metabolism of a calorie deficient rat.

Replication of the experiments using fats of known origin in rations for which heats of combustion were determined indicated that while body sparing activity is inherent in all fats certain fats seem to possess the property to a somewhat greater degree than others (Table IV). Butterfat and lard each appear to be more effective than either pure

TABLE IV  
NITROGEN EXCRETED IN URINE BY PROTEIN DEPLETED RATS FOLLOWING CALORIC RESTRICTION OF A PROTEIN FREE CARBOHYDRATE RATION AND OF RATIONS CONTAINING VARIOUS INDIVIDUAL FATS<sup>a</sup>

Fat used in ration	Nitrogen excreted in urine (mg per 5 days)				Increments in urinary nitrogen (mg)		
	Period I adequate calories	Period II restricted calories	Period III restricted calories	Total 10 days Periods II and III	From Period I to Period II	From Period II to Period III	
None	198	448	774	1222	250	326	
Cottonseed oil	243	324	474	798	81	150	
Wesson oil	240	315	492	737	71	107	
Butterfat	190	277	355	632	87	78	
Lard	194	278	335	613	84	57	

<sup>a</sup> All of known composition except Wesson oil it was purchased in local market

cottonseed oil or a Wesson oil purchased on the local market and of unknown composition

The greater efficiency of these fats is not apparent immediately upon restriction of calories to 25% of the dry's needs (Period II) but builds up with continued maintenance on the low calorie diet. Thus butterfat and lard each maintain the increment in urinary nitrogen associated with the prolongation of restricted calorie feeding into Period III at a lower level than is the case when either low fat or 20% cottonseed oil rations are fed (Table IV). Also the total nitrogen excreted during the two periods of low calorie feeding (II and III) by rats receiving either butterfat or lard is about one half the quantity excreted by the rats on the low fat diet that excreted by animals fed cottonseed oil is about two thirds this amount.

But it should be noted that the total energy value of the diet influences the relative body sparing properties of specific fats. For example rations in which an oil represents the lipid constituent accelerate excretion of urinary nitrogen to a greater extent in Period I when full calorie diets are fed than is the case when the ration contains no fat (Table IV). These data are in accord with those depicted in Fig. 4. The samples of pure butterfat and lard used in these experiments do not show this accelerating action, catabolism being of the same order as in the carbohydrate fed rats.

Thus is a result of properties possessed by butterfat and lard under the conditions of adequate and restricted feeding imposed in this experiment the total effect of these fats appears to be somewhat greater than that of oils in depressing rate of catabolism.

### G. COMPONENTS OF THE FAT MOLECULE

The data presented in Table V may be interpreted to mean that both the saponifiable and nonsaponifiable fractions of a cottonseed oil of known origin are effective in reducing losses of body nitrogen under conditions of semi starvation and protein deprivation (Fox *et al.* 1959b). The nonsaponifiable material fed represented the first of three low pressure distillation fractions. The amount provided in the ration (1.25%) was equivalent to the nonsaponifiable substances present in the ration containing 20% of cottonseed oil. The saponifiable materials were in the residue remaining after the removal of the three distillation fractions. This residue was incorporated into the diet so as to provide the same quantity of saponifiable material as the 20% cottonseed oil ration.

Both fractions maintain the excretions of nitrogen in the urine in Periods II and III at levels markedly below those characteristic of the rats given the high carbohydrate diet in the same periods. Differences



## II PATHWAYS OF METABOLISM

### I Nitrogen

In a series of experiments in which the partition of nitrogen in urine was studied (Hoover 1950 Hoover *et al.*, 1949), rations made into slurries of the same caloric density and portions of equal volume administered by stomach tube in amounts that provided daily either 10 or 14 calories per 300 gm rat. Also, groups were fed the full caloric ration in Period II to permit comparisons in the same interval of experimental history. The fat fed was a 1:1 mixture of butterfat and lard.

Shifts in the relative amounts of the various nitrogenous constituents present in the urine suggest that the use of carbohydrate in a protein free diet introduces blocks in metabolic patterns (Table I). The calorie starved, protein deficient rat in the absence of dietary protein is unable to carry acidic intermediaries, that arise in the catabolic breakdown of amino acids, through the metabolic steps required for their utilization. The acidity of the urine was not measured but a 10 fold increase in the amount of ammonia excreted is indicative of an excessive elimination of acidic substances, possibly organic acids.

On the other hand, if the caloric value of the diet is adequate the presence or absence of fat in a protein free diet fed to rats partially depleted of body reserves of nitrogen does not seem to alter appreciably the pathways of nitrogen excretion (Table VI).

The deteriorating influence of the carbohydrate diet may be seen in the increase in the amount of creatinine nitrogen undoubtedly depicting irreversible breakdown of functional nuclei of the muscle.

### 2 Carbohydrate

Glucose tolerance data presented in Table VII indicate that animals fed the high carbohydrate diets providing no protein seemingly lack mechanisms important in the metabolism of carbohydrate even though the energy value of the diet is reduced. That the incorporation of fat in diets of adequate energy value preserves these mechanisms also is evident for utilization of glucose by rats receiving these diets proceeds at an approximately normal rate. But, when the caloric value of the diet is reduced the metabolism of the sugar is retarded. Thus not the fat content but the energy value of the diet seems to control the carbohydrate metabolism of these protein depleted animals (Hoover, 1950 Hoover *et al.* 1949).

The disturbance in the ability of the rats receiving restricted amounts of the low fat protein free diet to utilize carbohydrate may have a parallel in the failure of diabetic animals to handle this nutrient. It is known that diabetic like syndromes are associated with nutritive im-

TABLE VI  
NITROGEN BALANCE AND PARTITION OF NITROGEN IN URINE OF PROTEIN DEFICIENT RATS WHEN PROTEIN FREE DIETS CONTAINING OR DEVOID OF FAT ARE FED AT TWO CALORIC LEVELS IN THE SECOND 5 DAY METABOLISM TEST (PERIOD II)<sup>a</sup>

Energy value of diet in Period II	Protein free diet fed	Nitrogen balance (mg per 5 days)	Partition of nitrogen in urine (mg per 5 days)					
			Total	Urea N	All in toin N	Ammonia N	Amino N	Creatinine N
Not restricted ≈ energy value of food provided in Period I 58 Cal per day	20% fat	— 288	180	79	80	5	4	14
Not restricted ≈ energy value of food provided in Period I 58 Cal per day	Low fat	— 280	180	85	61	2	11	18
Restricted ≈ 1/4 of energy value of food provided in Period I 14 Cal per day	20% fat	— 514	459	330	71	42	9	14
Restricted ≈ 1/4 of energy value of food provided in Period I 14 Cal per day	Low fat	— 1465	1389	875	81	349	4	28

<sup>a</sup> The lipid constituent is a 1:1 mixture of butterfat and lard

tion Indeed it was observed 40 years ago that patients with diabetes mellitus fared better on high fat diets than on high protein or normal diets (Allen 1917, Newburgh and Marsh, 1920) It also has been shown that rats made diabetic with alloxan behave similarly (Burn *et al*, 1944) One interpretation may be that the diabetic organism has limited ability to synthesize fat—a deficiency that is compensated for by increases in fat intake This idea is supported by experimental work showing the possibility that a major function of insulin is to increase the synthesis of fat in the organism (Drury, 1940 Pauls and Drury, 1942)

TABLE VII

AVERAGE GLUCOSE TOLERANCES OF GROUPS OF RATS FED A STOCK RATION AND TWO LOW NITROGEN DIETS AT TWO LEVELS OF CALORIC INTAKES

Energy value of diet in Period II	Diet fed	Mg % Minutes after injection of glucose					
		0	30	60	120	180	240
<i>Ad libitum</i>	Stock control	90	252	230	178	135	112
58 Cal/300 gm rat/day	20% fat	93	290	241	208	157	108
58 Cal/300 gm rat/day	Low fat	130	308	282	251	202	162
14 Cal/300 gm rat/day	20% fat	150	305	281	238	209	176
14 Cal/300 gm rat/day	Low fat	140	290	272	243	190	170

### 3 Adrenal Cortical Activity

Adjustments made to the triple stress imposed by this experiment are complex and all must be taken into account in trying to understand the adaptations made by the experimental animal Recent studies in our laboratory show that hyperemic enlarged adrenal glands and low concentrations of adrenal cholesterol are coupled with the high excretions of urinary nitrogen characteristic of rats receiving limited quantities of the diet in which carbohydrate is the source of energy

Decreased concentration of cholesterol in the adrenal glands is believed indicative of increased adrenal cortical activity (Long 1947) It appears that the presence of fat in the low calorie, protein deficient diet lessens at least in part the stress to which the animal is subjected because the adrenal glands of rats receiving this diet have normal concentrations of cholesterol If the markedly lower values for adrenal cholesterol observed in the rats fed the high carbohydrate diet signify hyperactivity this in turn may explain the increased catabolism of protein that occurs when fat is eliminated from a protein free diet of in

adequate energy value. However it also is possible that the adrenal response is secondary to the stress of large losses of body nitrogen. Or again fat may provide precursor substances for the synthesis of adrenal hormones.

### I. ROLE OF METHIONINE

The effectiveness of methionine in the sparing of body tissue of the rat when added to a protein free ration containing 20% of fat was reported from our laboratories in 1947 (Brush *et al*, 1947). Nevertheless, the observation that methionine may serve in lieu of fat in averting the

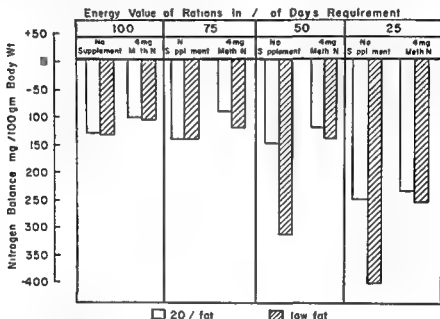


FIG 11 Nitrogen balances when protein free rations of varying fat content are fed to protein depleted rats at different caloric levels with or without methionine as a dietary supplement

catastrophic effects of feeding protein free diets of reduced caloric value to rats with low reserves of body protein came as more or less of a surprise. In these experiments methionine in a quantity equivalent to 4 mg of nitrogen each day was offered to rats at the beginning of the second metabolism period.

The data depicted in Fig 6 show that methionine in amounts providing only 4 mg of nitrogen per day exerts some protective influence on metabolism when supplementing either of the low nitrogen basal diets when calories are adequate (Hoover 1950 Hoover *et al* 1949). But it is even more potent under conditions of caloric restriction. It behaves like fat in depressing the excretion of urinary nitrogen when the food consumed provides only one fourth of the needed energy.

The ameliorating influence of methionine in rats fed the various protein free diets used in these investigations can be detected in various phases of the metabolism

Its presence in the diet is associated with a reduction in the excretion of urea by rats fed restricted amounts of either the 20% fat or the low fat diet (Fig 7) but is particularly effective in preventing the tremendous outpouring of urea that occurs when dietary fat is absent. Very interestingly, although dietary methionine alleviates the high production

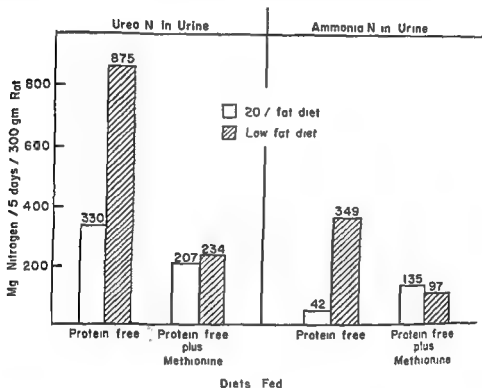


FIG 7 Urea nitrogen and ammonia nitrogen in urines of rats fed protein free diets of restricted value (14 calories per 300 gm rat per day)

of ammonia by rats in the low fat group it augments its production in rats receiving fat. As a result the proportion of urinary ammonia to urea nitrogen remains abnormal during methionine feeding in both groups on the restricted feeding regime.

Dietary methionine appears to modify the course of the carbohydrate metabolism under the conditions of the present experiment. It may be significant that it does not seem to exert its full regulating effect unless fat is present in the diet. Hoover for example has shown with glucose tolerance tests (Hoover 1950 Hoover *et al* 1949) that when methionine is added to the full calorie protein free diet containing fat the average glucose tolerance curve matches that of normal rats fed the regular stock

colony ration. But of greater interest is the fact that supplementary methionine nearly completely eliminates the partial block in carbohydrate utilization that occurs when this same fat containing diet is fed in amounts providing only one fourth of the needed calories. Dietary methionine also improves utilization of carbohydrate in the animal deprived of fat and receiving a restricted diet.

These observations in relation to the carbohydrate metabolism make attractive the hypothesis that the retardation in nitrogen catabolism induced by supplementary methionine reflects an improvement in utilization of carbohydrate for it has been shown that sulfhydryl compounds alleviate experimentally induced diabetes (Griffiths 1950, Lazarrow, 1946).

On the other hand methionine may act in some other capacity being important as a whole molecule rather than by virtue of constituent groupings. As such it may enter a key place in some enzyme or hormonal system. We for example have observed that ethionine acts like an antagonistic substance when it replaces methionine as a dietary supplement increasing the total catabolism with the excretion of urea remaining constant (Turnbull 1948).

But we cannot lose sight of the possibility that methionine and fat may be contributing independently to the production of certain body constituents the phospholipids being an important example. Ordinarily, the diet provides precursors for their synthesis. But in the stress of combined protein and calorie restriction metabolic patterns may be changed so that excessive requirements for these precursor substances are created. Choline although present in the ration may fall in this category also.

#### IV SUMMARY AND COMMENTS

That the energy producing components of the diet have a role in promoting utilization of food protein and in protecting the integrity of body tissue in the adult animal is clear. But no one metabolic picture describes the response of an experimental animal to variations in food energy value since the protein metabolism is a function not only of the energy value of the diet but of other simultaneously operating factors.

Whether catabolism or anabolism predominates in the adult animal following caloric restriction depends on the degree to which the energy value of the diet has been reduced, the quantity and nutritive value of the dietary protein, the size and nature of labile body reserves in respect to protein, fat and glycogen and in some instances dietary sources of energy.

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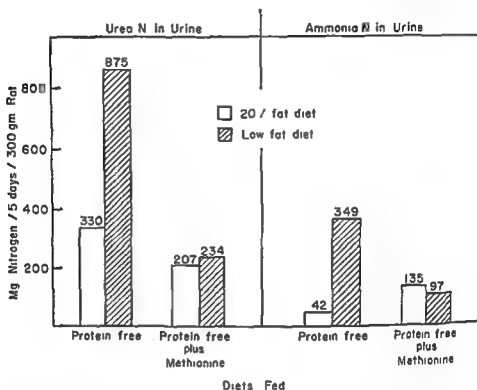


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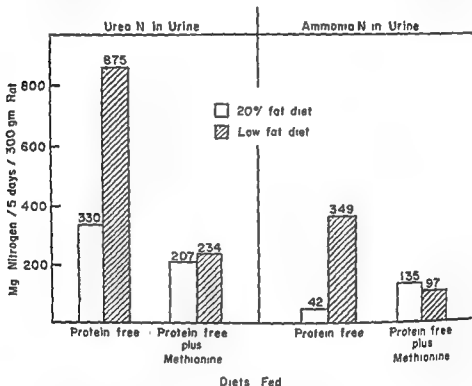


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appreciation of the interdependence of nutrients in nutrition has developed. And, as is the case in regard to the total energy value of the diet, the response of the animal to either the dietary inclusion or omission of either nutrient reflects physiological, dietary, and environmental conditions.

In general, when normal adult animals are used in experiments of sufficiently long duration, carbohydrate and fats supplied in adequate mixed diets exert equivalent effects on the utilization of protein and the retention of nitrogen. They behave similarly also upon restriction of the energy value of the diet, providing that the lipid content of the fat-containing diet is held within reasonable limits. This response has been called the nonspecific effect of calories upon nitrogen retention. But carbohydrate may also exert a specific effect upon protein utilization, as shown by feeding carbohydrate and fat simultaneously with and apart from, the protein moiety of the ration.

However, carbohydrate and fat are not equally effective in the maintenance of the endogenous nitrogen metabolism when a stress in the form of restricted food energy is imposed. Fat exerts a definite sparing effect, as shown by decreased excretions of nitrogen in the urine and deferred death. Its protective influence is demonstrable in rats adapted to protein-free diets and fed rations of low energy value, either containing or devoid of fat.

As the caloric value of the diet is progressively decreased, the significant role played by dietary fat in reducing the rate of catabolism becomes apparent. The incorporation of fat in the ration prevents the marked increase in the quantity of nitrogen excreted in the urine that occurs when carbohydrate provides calories in one-half the needed amount. When fat is present, there are no increments in urinary nitrogen until the calories are restricted to one-fourth of the requirement, and then increases are of definitely lower order than they are when carbohydrate is the source of energy.

But it is important to note that when the period of protein deprivation is prolonged without restriction of calories, fat loses its protective influence, as measured by rate of catabolism and time of survival. Thus the effectiveness of fat in sparing the endogenous metabolism appears to be a function of the amount of energy provided by the protein-deficient diet.

In regard to the efficiency of fat in sparing body protein, the quantity of fat present in the calorically restricted diet is important. Lipid constituents should represent at least 15% of the ration if the rapid catabolism associated with the feeding of low-fat, low-calorie rations is to be retarded.

Also individual fats seem to possess the nitrogen sparing properties in varying degree. Although all fats will retard nitrogen catabolism solid fats seem to have a greater protective effect than the oils especially in tests of long duration. In general it is believed that even though dietary fats and combinations of lipid components may differ in their effectiveness in depressing protein losses the presence of fat per se seems to be of greater importance than structural characteristics. If any fraction of the fat molecule possesses greater activity than another the nonsaponifiable portion in the case of pure cottonseed oil would be so designated.

The nature of the adaptation associated with prolongation of life, of which the protein depleted rat is capable when fed fat containing diets of low caloric value is not clear at the present time. Since the fat containing diet has been consumed or administered in quantities isocaloric with rations devoid of fat it seems that its protective role must be described in terms other than the provision of calories in this instance.

This role undoubtedly bears an intimate relation to the physiological state of the animals used in the experiments i.e. rats with greatly reduced stores of labile body protein. Normally enzymes represent a large part of the labile protein reserves in the liver (Miller 1948). With depletion concentrations of many tissue enzymes are altered so that imbalances in proportions of one to another arise (Allison 1957). Also there may be inhibition in the production or activity of vital hormones (Samuels 1946).

The animal body has mechanisms of adaptation to wide variations in the intake of the three major foodstuffs (Samuels 1946, Russell 1957). As a result many adaptations have occurred before caloric restriction is initiated. Thus the fat fed rat may enter the period of caloric stress with a body better equipped to handle the emergency than does the carbohydrate fed rat. Preliminary experiments suggest that dietary fat prevents the increase in cytochrome oxidase activity that has been associated with an absence of fat in the diet (Swanson and Artom 1950, Kunkel and Williams 1951) and with inadequate protein (Allison 1957).

How then in this experimental situation does fat function in retarding body breakdown? At this point we can only conjecture. In some way the fat component of the diet seems to confer economy of utilization of food energy. It may be significant that utilization of carbohydrate is approximately normal in rats fed fat in diets that meet the energy needs. Closely associated is the observation that dietary fat promotes the transformation of intermediate nitrogenous catabolites to urea and eliminates in large part the necessity for excretion of some of these intermediates as ammonium compounds.

The nature of the carbohydrate metabolism is being explored further

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The nature of the adaptation associated with prolongation of life of which the protein-depleted rat is capable when fed fat-containing diets of low caloric value is not clear at the present time. Since the fat-containing diet has been consumed or administered in quantities isocaloric with rations devoid of fat, it seems that its protective role must be described in terms other than the provision of calories in this instance.

This role undoubtedly bears an intimate relation to the physiological state of the animals used in the experiments, i.e., rats with greatly reduced stores of labile body protein. Normally, enzymes represent a large part of the labile protein reserves in the liver (Miller 1949). With depletion, concentrations of many tissue enzymes are altered so that imbalances in proportions of one to another arise (Allison 1957). Also, there may be inhibition in the production or activity of vital hormones (Samuels 1946).

The animal body has mechanisms of adaptation to wide variations in the intake of the three major foodstuffs (Samuels 1946; Russell 1957). As a result, many adaptations have occurred before caloric restriction is initiated. Thus, the fat-fed rat may enter the period of caloric stress with a body better equipped to handle the emergency than does the carbohydrate-fed rat. Preliminary experiments suggest that dietary fat prevents the increase in cytochrome oxidase activity that has been associated with an absence of fat in the diet (Swanson and Artom 1950; Kunkel and Williams 1951) and with inadequate protein (Allison 1957).

How, then, in this experimental situation does fat function in retarding body breakdown? At this point we can only conjecture. In some way the fat component of the diet seems to confer economy of utilization of food energy. It may be significant that utilization of carbohydrate is approximately normal in rats fed fat in diets that meet the energy needs. Closely associated is the observation that dietary fat promotes the transformation of intermediate nitrogenous catabolites to urea and eliminates in large part the necessity for excretion of some of these intermediates as ammonium compounds.

The nature of the carbohydrate metabolism is being explored further

in experiments in which the rate of acetate  $2\text{C}^{14}$  was determined under the various experimental conditions.

As adaptive processes shift in the deficient rats with the experimental interval certain fats prevent further excretion of urine nitrogen. This observation suggests that itself or a component grouping or some associated metabolic event may have a place in the regulation of important body precursor material for the synthesis of such substances. Or if dietary fat does not participate in suboptimal amount of some enzyme produced perhaps in suboptimal amount the manufacture of such substances for handling intermediate products may be established.

Again the fact that methionine when added to the low fat diets affects the general course of nitrogen catabolism in the as fat suggests that control of the lipid metabolism may be a factor. A need for precursor substances for the synthesis of protein may have been created by the triple stress condition with methionine each capable of supplying the critical intermediate.

Thus for the data describing the role of fat in the metabolism of protein deficient rat fed food of restricted energy value are descriptive. We have been attempting to portray the complete phenomenon can we explain the various adaptive changes of the endogenous metabolism. Suffice to say that probably the protein and fat metabolisms all are implicated. What the relationships may be will give insight into the adaptations of which organism is capable under the conditions of stress to which these particular animals were exposed.

#### ACKNOWLEDGMENTS

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## CHAPTER 9

# Methods of Measuring the Nutritive Value of Proteins, Protein Hydrolyzates, and Amino Acid Mixtures The Repletion Method

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## I INTRODUCTION

Determination of the nutritional value of proteins is inherently non-specific. In the case of vitamins or essential minerals one deals from the assay standpoint with the evaluation of a single essential nutrient at a time. In the assay of protein value conversely one must be concerned

with the qualitative and quantitative adequacy of at least 9 amino acids. There are also problems of amino acid balance and utilization to be considered. Thus, although blood carries amino acids to all tissues only one of the proteins of blood is nutritively balanced. The keratins of skin, feathers, and hair on the other hand, which have fair balance of amino acids are largely indigestible.

It is inevitable that different assay methods differ in classifying proteins as to nutritive value. The different assay methods have differing parameters of measurement and quite different endpoints. Despite the vagaries inherent in this type assay protein evaluation methods have classified most food proteins in the same general order of adequacy. The balanced proteins of meat, milk, and eggs have been used as standards of excellence. By the same token the major grain proteins, low in one or more essential amino acids are generally improved in feeding value by appropriate amino acid fortification. Balance of amino acids is usually achieved in man by a sufficiently varied food intake. Balance can also be purposefully achieved, as in the case of manufactured feeds for poultry and swine, by minor fortification of a single protein source, soybean meal.

We now know the approximate amino acid needs of several species. Knowing the essential amino acid composition of a protein one can predict its feeding value with fair accuracy. One cannot predict, however, failures in digestibility or effects of processing, some of which are not revealed by composition analyses. Protein methods which reflect true feeding value continue therefore to provide the ultimate in biological evaluation. Indirect methods of protein evaluation confirm and complement the direct feeding methods.

The pattern of amino acid requirements is much more similar than dissimilar for growing rats (Rose *et al.* 1948), for adult man (Rose 1949), for adult protein depleted rats (Cannon 1948), for adult rats (Benditt *et al.* 1950), and is exemplified in the National Research Council nutrient requirements for swine (Natl. Acad. Sci., 1953) and for chickens (Natl. Acad. Sci., 1954). Albritton (1954) has compiled requirements also for mice, turkeys, dogs, various invertebrates, and microorganisms insofar as these are known. In the search for "unity amid diversity" the nutritive quality of proteins has equal connotation for many species. Muscle and tissue proteins differ little as to amino acid composition between species. Generally choice of one protein evaluation method over another is made on the basis of convenience or application to a given species, or set of circumstances.

Meister (1957) has provided an excellent treatise on the biochemistry of amino acids including their nutritive role. The nutritive value of pro

teins was recently reviewed in a colloquium of six papers (Cuthbertson 1957). One of these by Bender reviews recent biological methods of evaluating protein quality. Bender describes among others his own abbreviated protein retention assay (Bender and Doell 1957) the 2 day nitrogen restitution method of Vardi and Tatar (1954) the single dose nitrogen retention test of Silber and Porter (1949) for protein hydrolyzates and the microbial assays of Fernell and Rosen (1956) Hilevy and Crossowicz (1953) and Horn *et al* (1952). Only the methods of Bender (1956) and Bender and Doell (1957) are reviewed here. In this same colloquium both Carpenter and Illinger detail the accomplishments and many of the limitations consequent to processing proteins for use in animal feeds. Importantly also Henry and Kon (Cuthbertson 1957) strive to bring the whole matter of protein evaluation into perspective pointing the need for reliable assessment of diets as eaten.

In an earlier review of the same title (Frost 1950a) classic methods of protein evaluation were discussed pertinent to evaluation of the rat repletion method. The latter was new at that time and was extensively applied to assay liquid protein hydrolyzates. Weanling rats cannot consume enough highly dilute protein hydrolyzate solution to support maximum growth. Neonatal protein depleted rats if need be consume volumes approaching their own body weight each day. The speed and precision of the method plus the fact that the same animals are used for repetitive assays serving to some degree as their own controls proved uniquely valuable for this purpose.

The basic repletion method was developed as a tool by Cannon (1945) in his classic studies of the pathological consequences of protein deficiency particularly the loss of natural resistance to disease. The method itself proved so intriguing that Cannon and his students went on to establish the essential amino acid requirements to support rapid repletion in the adult protein depleted rat. This provided the same precise basis for assay of proteins by the repletion technique as Rose and his co-workers (1945) had established for growth. Cannon (1954) reviewed the advantages and scope of the rat repletion method describing its flexibilities and broad application to medical problems.

In the earlier review questions were raised as to the effects of amino acid balance the role of other than essential amino acid nitrogen and the inhibition by D-amino acids. The nature and consequence of amino acid imbalance is rapidly unfolding (Elvehjem 1956 Harper 1958). The same is true for effects of D-amino acids (Berg 1953 Wretling 1956 and Womack *et al* 1957). These are areas in which the repletion method can be used conveniently with precision and at relatively low cost.

Fisher (1956) has raised the interesting question whether excessive intake of essential amino acids which are all quite toxic given individually may limit life span. This is a timely question to which Fisher suggests an experimental approach. We can only conjecture now, however, on such points. Presumably the highest nutritive value proteins place least metabolic stress.

Studies in rats at the National Institutes of Health with chemically defined water soluble diets (Greenstein *et al.*, 1957) clearly show that mixtures of only the natural L amino acids are indeed superior for growth in rats to ones containing various DL amino acids. As previously noted (Frost and Sandy, 1950) the avidity of protein depleted rats for mixtures containing DL amino acids was always markedly less than for protein hydrolyzates. This was true even when a two fold excess of essential amino acids was supplied. Sources of nitrogen other than essential amino acid nitrogen such as glycine, glutamic acid, arginine, ammonium salts, and even urea proved superior to an excess of essential amino acids. Because the mixture of essential amino acids contained some DL forms at least part of the inhibition was suspected due to the D amino acids. Recent studies by Rechcigl *et al.* (1957) bear on this point and, like the reports of Greenberg and his co-workers, reveal the precision in interpretation which is only possible when L amino acid mixtures are used.

In time re-evaluation of the essential amino acid requirements for repletion should be made in terms of only the natural isomers. When this can be done it will again be worth while to determine the substitution value of various nonspecific forms of nitrogen.

In a precise series of investigations Schultze (1956) has shown that chemically defined diets suffice for reproduction in rats. The 10 "essential" amino acids did not support lactation as well as a mixture of 16 amino acids, similar to the findings for both growth and repletion in rats. This work supports the general notion that the nutritive quality of proteins is reflected also in their ability to support reproduction and lactation.

## II. PROTEIN EVALUATION METHODS — GROWTH AND NITROGEN BALANCE

### A. GROWTH METHOD AND PROTEIN EFFICIENCY RATIO

The way in which different proteins support growth in young rats has been the most general and widely used criterion of protein value. In 1919 Osborne and his co-workers introduced the concept of "protein efficiency ratio" as a refinement of the simple growth method. The grams weight gain per gram protein intake were measured for several proteins

and it was found that varying levels of protein in the diet gave different protein efficiency ratios. A rather definite level was found for each individual protein which produced the greatest gain per gram protein ingested. These levels for casein and lactalbumin were 12% and 7.9% respectively. In general it has been found that the better the protein the lower the level in the diet required to produce the highest protein efficiency ratio. This is a clear reflection of the importance of the proper nutritive balance of all of the amino acids to produce optimum metabolic efficiency. In practice however it became fairly customary to determine protein efficiency ratios at a level of 10% of dietary protein. Mitchell (1944) criticized the method on the basis of certain of the weaknesses which were recognized by its originators.

Burns and his associates working with mice made a careful study of the protein efficiency method which led Bosshardt *et al* (1946) to a new improved and shortened technique. It may be mentioned again however that Block and Mitchell (1946) have criticized these refinements in the method as being too cumbersome to be generally followed. The latter criticism may of course be voiced for the nitrogen balance methods as well depending on one's experience and accommodations. Hegsted and Worcester (1947) appear to have brought the most cogent criticism of the protein efficiency ratio method as ordinarily practiced. They have established that in a large series of experiments the protein efficiency ratios almost exactly parallel the rates of weight gain. Thus, in the ordinary comparison of proteins by the growth method nothing may be gained by the calculation of protein efficiency ratios over and above the information obtained from direct comparison of the growth increments themselves and the extra effort of measuring daily food intakes is avoided.

Nitrogen efficiency ratios have been calculated in this laboratory under the conditions of the rat repletion method wherein the rats are allotted a definite amount of amino acid nitrogen daily. In instances where rats do not take the complete allotment of nitrogen the figure for nitrogen efficiency ratio provides a useful reflection of the net nutritive values of various preparations. In instances where animals consume the full allotment of nitrogen comparison of weight increments alone is sufficient. It may be mentioned that the basis of feeding levels used in this laboratory is nitrogen rather than protein. This seems appropriate in view of the considerable difference in nitrogen content of different proteins and even between different samples of the same protein.

The growth method of assay obviously classifies proteins according to their adequacy to meet the needs for tissue synthesis and is generally considered a critical index of protein value. The method is most critical

Fisher (1956) has raised the interesting question whether excessive intake of essential amino acids which are all quite toxic given individually, may limit life span. This is a timely question to which Fisher suggests an experimental approach. We can only conjecture now, however, on such points. Presumably the highest nutritive value proteins place least metabolic stress.

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pointed to the independence of the conversion of creatin to creatinine by dietary amino acids as a case in point. Berg (1948) has resolved the argument over the reality of endogenous nitrogen and concludes as follows: "The change needed is one of viewpoint. What was once considered static is now proved only to have appeared so. The state which actually obtains is one of equilibrium involving the summation of a complex array of interdependent equilibria between opposing dynamic forces." Murlin *et al* (1948) have made the interesting observation that the per cent of total nitrogen excreted as creatinine varies for different proteins and that close correlation can be drawn between this percentage figure and the biological value of the protein fed. Creatinine nitrogen is remarkably constant on a creatine and creatinine free diet and this constancy adds workable credence to the concept of "endogenous nitrogen excretion."

#### D EXPERIENCE WITH THE NITROGEN BALANCE METHOD

Early in the work on intravenous protein hydrolyzates in this laboratory, Risser *et al* (1946) concluded on the basis of short term nitrogen balance studies in dogs that there was no difference in the biological value of partial acid hydrolyzates of both casein and fibrin over a wide range of hydrolysis extending to complete hydrolysis. Later work in which dogs were maintained over very long period of time (Frost and Risser 1946) and in which severely protein depleted dogs were used (Frost *et al* 1946) led to somewhat different conclusions and demonstrated the need for considerably more heroic measures than the simple 4 or 5 day nitrogen balance method to provide a final answer. Results of this study are shown in Fig. 2.

Allison and co workers (1949) reported that wide variations in the degree of enzymatic hydrolysis of casein did not significantly alter the nitrogen balance index as determined in 4 day periods. The dogs were consistently in negative balance at an intake of 120 mg. nitrogen per kilogram per day. Lactalbumin was fed during the 3 day periods before and after injection. The findings are comparable to the early findings in this laboratory except that our dogs were essentially in balance. Our subsequent findings however would cast much doubt on the real significance of such short term experiments.

We may also make general note of the experience of the six laboratories which collaborated in the U. S. P. study of the nitrogen balance method for the standardization of intravenous protein hydrolyzates. The study was carried out under the chairmanship of J. B. Allison and the author and the method used was essentially that of Allison *et al* (1947). The results of the general study showed great variation both



fed animal requires much more nitrogen from a given source to maintain equilibrium than does the depleted, or standardized, animal. The well animal remains in obvious health and buoyant spirits, and in so doing, appears quite prodigal of food nitrogen. The depleted animal on the other hand, has great avidity for nitrogen and this avidity is not ordinarily satisfied except by a prolonged period of high protein feeding. This situation is difficult to reconcile with the exact analytical requirements of the nitrogen balance methods as applied to the assessment of protein value.

### 3 Egg Replacement Method

This method is essentially the determination of biological value as proposed by Thomas and by Mitchell, and has been carried out chiefly by Murlin and his co-workers in studies with adult humans at Rochester. Sumner *et al.* (1938) observed that egg protein is of outstanding value for maintenance of nitrogen balance. Egg protein was found to have a biological value near 100; that is, the protein was almost completely absorbed and there was no urinary loss of nitrogen over the normal loss on protein free diet. This led to the concept of determining "biological value relative to egg." It also led to the practice of standardization of test subjects on maintenance levels of a high biological value protein, rather than on nonprotein diet. Values for "endogenous nitrogen excretion" obtained when the subject is on a very low level of protein are certainly as reliable as values obtained when the subject is on nonprotein diet. Furthermore, protein depletion is avoided by the first procedure. The last of a series of six papers from the University of Rochester group (Hawley *et al.* 1948) is concerned with the determination in adult humans of the biological value of the six reference proteins distributed from Rutgers University (1946-1950).

### C ENDOGENOUS NITROGEN

It is generally agreed that Folin's theory of endogenous and exogenous protein metabolism is no longer tenable *in toto*. Borsook (1950) has recently reviewed the question of protein turnover and rate of amino acid utilization. As he points out, it has become increasingly clear that the rate of turnover of body protein in animals in nitrogen balance is far greater than was envisaged by Folin's theory. The effects of endocrine stimulation of nitrogen excretion and retention are dynamic and may at any time serve to jeopardize the value of nitrogen balance determinations.

Block and Mitchell (1946-47) have defended the concept of endogenous nitrogen as applied in the calculation of biological value and have

pointed to the independence of the conversion of creatin to creatinine by dietary amino acids as a case in point Berg (1918) has resolved the argument over the reliability of endogenous nitrogen and concludes as follows "The change needed is one of viewpoint. What was once considered static is now proved only to have appeared so. The state which actually obtains is one of equilibrium involving the summation of a complex array of interdependent equilibria between opposing dynamic forces." Murlin *et al* (1948) have made the interesting observation that the per cent of total nitrogen excreted as creatinine varies for different proteins and that close correlation can be drawn between this percentage figure and the biological value of the protein fed. Creatinine nitrogen is remarkably constant on a creatine and creatinine free diet and this constancy adds workable credence to the concept of "endogenous nitrogen" excretion.

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within and between laboratories although some classification of the different products could be made in a broad way. The study clearly demonstrated the need for careful standardization of the test animals but failed to provide a highly critical and practicable method of standardization. A primary criticism of the method was the inadequacy of the 4 day period to allow a good resolution of differences.

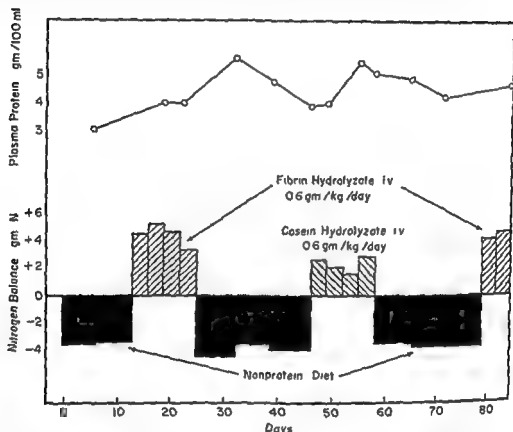


FIG 2 Graphic presentation of the sequence of repeated protein deprivation on nonprotein diet and regeneration on massive intravenous therapy. The graph corresponds to the data mentioned in the text (Frost *et al* 1946) and shows the method of comparing the ability of two different hydrolyzates to correct severe experimentally induced hypoproteinemia.

A further point of interest is found in the report of Silber and Porter (1949) who have described the results of their own laboratory in the above collaborative study. These authors reported a zero biological value for one preparation when injected as opposed to good utilization when the product was given orally. The general conclusion was that there was a significant loss of peptides following injection of all partial hydrolyzates and that certain products were markedly less well utilized by injection than by oral dosing. These results are quite contrary to those of Allison *et al* (1947). In the authors' opinion it is rather fruit

less to draw final conclusions on such points of difference without recourse to more rigorous criteria such as long term balance studies at minimum nitrogen levels and the correction of induced hypoproteinemia. As shown by Weech (1942) the rate of plasma protein regeneration is a useful criterion of protein value in the latter type study.

In the experience in this laboratory the short term method of determining biological value or nitrogen balance index in adult dogs is not as useful for resolving differences in protein and protein hydrolyzate values as are the rat repletion or rat growth assay methods. The large quantitative differences between requirements for growth and maintenance may account in part for the differences in discerning power of the two types of assay. Hegsted *et al* (1945) have estimated that the tryptophan requirement per kilogram body weight for growth of rats versus nitrogen balance in dogs are 94 and 16 mg respectively and for isoleucine 880 and 15 mg respectively. In the normal adult animal there is clearly a reserve however small of protein which makes for equilibrium. In the depleted or growing animal conversely there is no reserve and all of the driving mechanisms of the body are in the direction of protein storage. The quantitative day to day requirements for equilibrium are not nearly so critical as are those for growth or repletion.

## E. NET PROTEIN UTILIZATION AND PROTEIN RETENTION EFFICIENCY

Miller and Bender (1955) describe a 10 day method to estimate that fraction of the food nitrogen eaten which is retained by weanling rats. The net protein thus used (NPU) is calculated from a formula which like the calculation for nitrogen balance accounts for the amount of nitrogen needed for maintenance. A group of rats on nonprotein diet provides the endogenous or maintenance N correction. The body nitrogen deposited as new tissue by the test group is thus estimated with reference to the total nitrogen consumed. Body water is determined in place of body nitrogen in the shortened method. This procedure is based on the assumption that the ratio of nitrogen to water is constant. In various assays calculation from the water content of rats appeared to agree well with values calculated from nitrogen content.

In a careful comparison of the above method with measurement of protein efficiency ratio Bender (1956) reveals certain limitations of the latter. Thus rats accepted various amino acid mixtures to varying degree. This failure of acceptance has a more profound effect in calculating protein efficiency ratio than for net protein utilization. Despite this variation values obtained by the two methods for various proteins agreed well.

Bender and Doell (1957) recently describe a further modification of the method for protein efficiency ratio, as follows: paired litter mate groups are fed 10% of a test protein or nonprotein diet for 10 days; the difference between the gains in weight of the two groups divided by the weight of protein eaten is termed net protein ratio; this value, multiplied by 16, is called the protein retention efficiency. The latter encompasses the maintenance needs of the test animal as well as the growth requirement. Protein retention efficiency correlates in turn with values determined by carcass analysis for net protein utilization. As final conclusion the authors state: "It is concluded that body weight is a reasonably accurate index of body protein in young growing rats."

This series of investigations thus in a sense completes a full circle to reveal that measurement of growth rate alone may suffice to differentiate protein value. If indeed, growth rate closely parallels protein efficiency ratio (Hegsted and Worcester, 1947), little may be gained in practice by these more complex methods.

Rate of growth at low protein level clearly classifies proteins as to adequacy, balance, and availability of amino acids. Various protein feeding levels can be used with the growth technique. This helps to establish the extent of the primary limitation. When the essential amino acid composition is known, one can readily estimate which amino acids limit growth. Stepwise amino acid fortification then completes the picture. Calculation of protein efficiency may provide a useful check, particularly in instances where failures in digestibility or absorption are suspected.

Although Bender (1956) studied response to amino acid mixtures, no attempt was made to establish the method for net protein utilization in terms of precise amino acid needs. One may conjecture that failure of rats to consume the amino acid mixtures used was due to inhibition by a few amino acids. Here again, assay, based on response to only a few amino acids, would have finite value.

Interpretation is difficult and one can easily become immersed in semantics when too many methods and too many slightly ambiguous terms are compared. Bender (1958) in measuring the nutritive value of bread protein attempts to correct errors in interpretation made by other workers. Despite these differences, three investigations came to the similar conclusion that about half of the protein of bread is used for growth and that utilization is increased at least 10% by addition of lysine.

### III ASSAY METHODS BASED ON PROTEIN REGENERATION

#### A REGENERATION OF LABILE LIVER CYTOPLASM

Addis and his collaborators (1940) examined the relationship between dietary protein intake and protein content of the liver and found it to be a fair measure of protein value. Kosterlitz and Campbell (1945) developed rapid methods based on the estimation of labile liver cytoplasm following brief fasting in rats and Harrison and Long (1945) confirmed the general method. The methods are based on the fact that adult rats fed protein free diet lost practically all labile liver cytoplasm in a few days and that the rate of regeneration is a fair measure of the nutritive value of a test protein. The method has been examined statistically and reduced to as simple a form as possible by Campbell and Kosterlitz (1945). The method even in simplest form however requires several determinations on the excised livers of highly standardized animals and therefore appears somewhat formidable as compared with methods based directly on body weight gain under equally closely defined conditions.

Mendes and Waterlow (1958) have reviewed the recent literature on the effects of protein deprivation on the deoxyribonucleic acid (DNA) content of tissue. They determined that a low protein high carbohydrate diet, simulating that eaten by the poor people of Jamaica, completely arrests growth in weanling rats. The ratio of nitrogen to DNA was reduced in both liver and muscle. On re feeding new protein and DNA appeared rapidly in the liver more slowly in muscle. Although this technique is not posed as a protein assay method it was suggested as a method to help assess the effects of protein malnutrition in human infants.

#### B MAINTENANCE OF PROTEIN DEPLETED RATS

Tomarelli and Bernhart (1947) have described a method which measures the amount of nitrogen from any protein which is required to maintain constant weight in protein depleted rats. The method calls for a one week depletion of adult rats on nonprotein diet. Separate groups are then fed varying levels of the test samples and standard protein and the effects on weight are observed for one week. The weight change plotted against nitrogen intake provides an index to maintenance requirement which is evaluated by comparison with the casein standard. Neither of the above methods have been widely studied. Nor have they been standardized in terms of the requirement for each of the individual essential amino acids. Both methods appear nevertheless to offer certain advantages in special situations which may recommend them for more thorough investigation.

## C REPLETION METHOD

Cannon *et al* (1944) devised a fairly rapid method of protein assay as a tool in their studies of the relationship of protein metabolism to antibody production and resistance to infection. The latter relationships and the pathologic consequences of protein and amino acid deficiencies have been reviewed by Cannon (1948, 1945). As pointed out by Cannon, the principle utilized is similar to that used often in biology, viz., the production of a biological deficit in order to measure the replacement value of a material to be tested. The method can demonstrate variations in protein quality in 1 to 2 weeks. Adult protein depleted rats show great avidity for protein supplements, and typify the clinical conditions wherein the use of protein and balanced amino acid feeding is most clearly indicated. According to Cannon (1945) also, the method has shown excellent agreement with the rat growth assay in unpublished experiments for the Quartermaster Corps.

In the early work (Cannon *et al* 1944) on the rat repletion method a close parallelism was noted in the capacity of proteins to promote both weight recovery and plasma protein regeneration. The specific effects of the essential amino acids for the two functions were exactly shown in two concurrent papers by Frazier and his co-workers (1947) and by Benditt and associates (1947). In the first paper, the requirement in the adult rat for each of the 9 amino acids, previously shown by Rose to be essential to the growing rat, was established. The omission of any one of the 9 essential amino acids led to prompt loss in appetite and weight. The promptness and clear cut effects on appetite and weight gain upon restoration of the individual missing amino acids likewise leave no doubt as to their essentiality and profound physiological effect. The findings clearly demonstrated also the surprising fact that food acceptance by protein depleted rats may be determined by the presence or absence of only a few milligrams of a particular essential amino acid. Cannon (1948) has tabulated the approximate daily requirement in milligrams for rapid weight recovery for each of the 9 essential amino acids and has shown that the ratio of these requirements is very close to the ratio in which these amino acids occur in whole egg protein.

The requirements for maintenance and for repletion in the adult rat have been shown to be qualitatively similar by Wissler *et al* (1948). The quantitative requirements for maintenance of nitrogen balance and of weight have been reported by Frazier *et al* (1949). The ratios of these requirements are of interest and differ only in a few instances, one from the other as shown in Table I. Rose's (1937) minimum

TABLE I  
COMPARISON OF RAT REQUIREMENTS FOR THE ESSENTIAL AMINO ACIDS FOR GROWTH, REPLETION AND MAINTENANCE<sup>a</sup>

Amino acid	Repletion requirements <sup>c</sup>		Requirements for maintenance of weight <sup>b</sup>			Growth requirements <sup>e</sup>	
	mg./rat dry <sup>c</sup>	Ratio	mg./100gm.	day	Ratio	% of diet	Ratio
Tryptophan	14.5	1	2.2		1	0.2	1
Phenylalanine	45	3.1	6.0		2.7	0.7	3.5
Leucine	72.5	5	8.0		3.0	0.8	1
Isoleucine	61	4.2	13.7		6.2	0.5	2.5
Methionine	39	2.7	7.3		3.3	0.6	3
Threonine	43	3.0	5.3		2.1	0.5	2.5
Lysine	58	4.0	4.5		2.0	1.0	5
Histidine	21.5	1.4	2.2		1.0	0.4	2
Valine	51	3.5	10.0		4.5	0.7	3.5

<sup>a</sup> These values which are somewhat different from those first reported (Cannon 1948) are the preferred values and are used here through the courtesy of Dr. I. Aul Cannon and his collaborators (Steffee *et al.* 1950)

<sup>b</sup> Frazier *et al.* (1949)

<sup>c</sup> Rose (1937)



requirements for rat growth are also shown. The difference in ratios of requirements for the individual amino acids is not large enough in any of the three situations to be considered significant, except possibly in the case of lysine in which the ratio of requirement for growth and repletion is higher than for maintenance. These data provide a better foundation for the comparison of methods based on growth, repletion and maintenance than has obtained heretofore.

### 1. Assay of Dry Protein Hydrolyzates

The assay of various protein hydrolyzates by the rat repletion method has been reported by Wissler *et al.* (1947). The following points were listed by the authors as advantages of the adult protein depleted rat as a test animal to measure the potential value of protein hydrolyzates.

(1) Its qualitative essential amino acid requirements parallel closely those of man.

(2) Such animals simulate conditions of protein depletion seen in many sick patients.

(3) The small size of the rat and its ready consumption of synthetic rations make the assay relatively simple and inexpensive.

(4) Protein depletion provides an increased stimulus for the fabrication of tissue and blood proteins. Consequently in a relatively short experimental period, one can measure much larger increases in protein than those found in physiological growth.

(5) It is possible to measure refilling of several protein compartments simultaneously. The authors point out further that weight recovery alone is sufficient as a measure of protein value, as this parallels the regeneration of plasma protein, hemolysin hemoglobin, liver protein and total carcass protein.

The amino acid requirements of rats for growth, maintenance, and repletion may now be compared with maintenance requirements for the adult human. The ratios of requirements of Table I may be compared with the minimum requirements for maintenance of nitrogen balance in adult humans recently reported by Rose (1949). Comparison may also be made with the calculated human maintenance requirements of Harte and Trivers (1947) and of others. Albarnes (1947) has further estimated the amino acid needs of infants and of children. The latter values obviously provide a less secure basis for comparison than do the experimentally determined values. An objective should be to determine as exactly as possible the amino acid needs for growth in children and for optimum rate of protein regeneration in adult protein depleted humans; however, such a program is beset with great difficulties and will certainly not be accomplished in the near future.

As a point of interest in Table II we have compared the ratios of the rat repletion requirements of Cannon and the minima for nitrogen balance in humans established by Rose and calculated by Harter and Travers. It is important to point out certain of the pertinent differences in the way in which the respective minima were established as these differences have a bearing on certain of the requirements. For instance, the human minima (Rose 1949) were established in absence of cystine and tyrosine so that the methionine and phenylalanine requirements are absolute. The rat repletion requirements were established on the other hand with a 16 amino acid mixture (Frazier *et al.* 1947) containing small amounts of cystine and tyrosine so that the methionine and phenylalanine requirements are not absolute. In each case it would appear that there was sufficient nitrogen other than essential amino acid nitrogen to spare the latter fully for tissue synthesis. Thus the relative ratios of requirements for methionine and phenylalanine as determined for the human would be expected to be somewhat greater than that for the rat as is indeed the case.

Comparing the ratios of requirements in Tables I and II one has little to choose in selecting a method which is most representative of human needs. In general one would say that methods based on growth maintenance or repletion would be of equal value and that the choice of method may rest with the inherent and desired accuracy of the methods themselves.

## 2 Assay of Liquid Protein Hydrolyzates

The assay of liquid protein hydrolyzates presents a variety of problems as follows: (1) hydrolyzates containing dextrose cannot be conveniently dried without loss of nutritive value; (2) dried hydrolyzates are hygroscopic and, therefore, difficult to blend into dry rations; (3) weanling rats cannot or do not ingest sufficient volumes of dilute hydrolyzate solutions to meet the needs for maximum growth.

Mueller (1945) reported the assay of liquid protein hydrolyzates fed in dry or liquid form to weanling rats. Although the method was not eminently successful it provided the first working basis for a rat assay. Frost and Sandy (1948a, b) adapted the "rat repletion method" to the assay of liquid protein hydrolyzates in protein depleted rats. The method had a marked advantage over the growth method previously reported by Mueller in that adult protein depleted rats are able to consume very large volumes of liquid and show a much more uniform appetite for liquid protein hydrolyzates than do young rats.

The question of liquid balance and liquid tolerance is an interesting one in itself with regard to the way in which animals of various ages

TABLE II  
RATIO OF MINIMUM DAILY REQUIREMENTS OF THE INDISPENSABLE AMINO ACIDS AS DETERMINED BY THREE METHODS  
RAT REPLETION<sup>a</sup> NITROGEN BALANCE STUDIES IN ADULT MEN<sup>b</sup> AND CALCULATION FROM MINIMUM PROTEIN REQUIRE-  
MENTS FOR MAINTENANCE IN ADULT HUMANS<sup>c</sup>

Amino acid	Rat repletion requirement		Requirement for N balance in adult men		Calculated requirement for N balance in adult humans	
	mg /rat day	Ratio	gm /day	Ratio	gm /day	Ratio
Tryptophan	145	1	25	1	0.4	1
Phenylalanine	45	31	11	44	1.4	35
Leucine	725	50	11	44	1.7	42
Isoleucine	61	42	0.7	28	1.2	30
Methionine	39	27	11	44	0.5	13
Threonine	43	30	0.5	20	1.0	25
Lysine	58	40	0.8	32	0.8	20
Histidine	21	14	—	—	0.5	13
Valine	51	35	0.8	32	1.1	28

<sup>a</sup> Cannon (1948)

<sup>b</sup> Rose (1949)

<sup>c</sup> Harte and Travers (1947)

can and will take liquid nutrients. Robinson and Adolph (1943) have established that a deficit of water relative to other body components provides a signal which initiates drinking in ordinary life. The amount of water drunk to correct the immediate deficit is ordinarily about 0.5% of body weight. Under extraordinary urges to drink in order to obtain nutrients, however, adult rats may drink more than their own body weights of water every 24 hours. Adolph (1947) has reported that adult rats maintained weight on milk diluted to as low as 2.6% solids but only by drinking very large volumes sufficient to meet caloric needs. McCance and Wilkinson (1947) have made the further pertinent observation that very young animals are less capable of handling large volumes of liquid than adult animals. These combined observations suggest that the adult protein depleted rat is well adapted to the assay in question whereas weanling rats are not as well adapted.

Dutta and Thadani (1957) reported the repletion value of horse fibrin hydrolyzate greater than that of a similarly prepared acid hydrolyzate of casein or of Amigen. The method of Frost and Sandy (1948a, b) was used but with essentially *ad libitum* feeding of the hydrolyzates i.e. 50 ml per rat day. The standard error of the comparison was not reported thus leaving the comparison with Amigen open to question. Where comparisons of this type are undertaken one may urge assay at controlled levels of nitrogen input and full report of the data.

Suitability of the repletion method for assay of amino acid solutions was studied by Cannon *et al* (1950). They emphasized the speed with which catabolism ensues in acute deficiency of any one of the essential amino acids. They demonstrate also the sensitivity of the method to reveal slight amino acid imbalance.

### 3 U S P Method for Protein Hydrolyzate Injection

An advantage of the repletion method particularly from the control standpoint is that rats can be used repeatedly for as many as five assays. The "Pharmacopeia of the United States" (15th rev. ed. 1955) described the method as a standard of nutritive adequacy for Protein Hydrolyzate Injection. The method has been used for routine control of production lots of aqueous 5% protein hydrolyzate in our laboratory. The U S P method is modified so that water is supplied *ad libitum* throughout the assay. This has given less variation in rate of gain than restricting water intake, as outlined in the U S P method.

The method is a rapid, convenient and accurate means to determine nutritive adequacy and balance of amino acid mixtures and protein hydrolyzates in solution. As discussed elsewhere in this review the

method may not reflect the true intravenous feeding value of partial hydrolyzates of proteins. The method is more directly adaptable to complete hydrolyzates and amino acid mixtures. With appropriate allowance, correcting for peptide loss on injection, the method may approximate the true intravenous feeding value. A further complication, only recently recognized, is the apparent failure of oral assay to detect the presence of amino acid hexose reaction products. Further research is needed to establish the significance of this reaction both for initial sterilization by autoclaving and as induced by normal aging.

#### 4 Other Applications in Rats

Cox *et al* (1953) used the repletion method effectively to measure rate of recovery of rats fed suboptimal amounts of protein hydrolyzate dextrose mixture as compared to an isocaloric amount of dextrose. They showed that the greater need for protein the greater the use of protein hydrolyzate. This applied when calorie intake from dextrose was greater than 25% of estimated need. The implication was made by the authors that such response to protein hydrolyzates should apply equally for parenteral feeding. On the other hand, Christensen (1956) has questioned the full utilization of partial hydrolyzates given parenterally.

Data by Christensen *et al* (1946-1955) leaves little doubt that peptides and reaction products of partial hydrolyzates and particularly those with dextrose are excreted in part when given parenterally in humans. Unpublished experiments by Lambert and Frost in dogs are in accord. This limitation was not revealed in extensive studies by Overby and Frost (1952) using the rat repletion method. The latter work clearly showed incipient reactions in fibrin hydrolyzate heated with dextrose. In accord with the earlier report by Friedman and Kline (1950) increase in fluorescence was noted as perhaps the earliest evidence of complex formation. Decrease in nutritive value was not revealed however, until there was some disappearance of L amino nitrogen and also of tryptophan. These changes in turn did not occur until heating was sufficient to produce obvious browning of the hydrolyzate dextrose solutions. By repletion assay (Frederickson *et al* 1955) fibrin hydrolyzate with glucose lost nutritive value on heating faster than fibrin hydrolyzate with fructose. This loss appeared to parallel the rate of color formation.

As reported by Silber *et al* (1946) the metabolic fate of amino acid mixtures orally or parenterally may be similar. The evidence at hand suggests however that retention and use of complete hydrolyzates and amino acid mixtures autoclaved or aged with dextrose will favor the oral route. This problem may logically be resolved by admixing amino

acid and sugar solutions just prior to injection or by separate injection. Thus more research is needed to establish the optimum in parenteral amino acid feeding. The problem and the goal are, however, now well defined and wait technological accomplishment.

The acute metabolic disturbance which attends deficiency of potassium in protein depleted rats was first shown by Cannon *et al* (1951, 1952). This was clearly verified by Frost and Sandy (1953) who studied also effects of deprivation of sodium calcium phosphorus and magnesium on rate of repletion. Very little sodium appeared necessary under the test conditions. When phosphorus was withheld repletion was slowed but failure and death did not occur as with potassium deprivation. As with sodium deprivation of calcium or magnesium did not appear to limit recovery to any marked degree. Magnesium deprivation had greatest effect of these three. Requirement for potassium for maximum recovery was established at more than 7 mg—up to 14 mg potassium per rat day.

Allison and Wannemacher (1957) point to the variety of metabolic effects which may be brought about by protein depletion. Scientific effects limiting antibody formation and phagocytosis are reviewed by La Via *et al* (1956). Failure of testosterone to influence rate of either depletion or repletion was reported by Geiger and El Rawi (1952).

Cannon's group at the University of Chicago have systematically applied the repletion method to various aspects of protein metabolism. For instance Dittmer *et al* (1950) showed the suitability of the protein-depleted rat for study of amino acid antagonists. The work in this case was done with beta 3 thienylalanine a phenylalanine antagonist. Use of intravenous fat emulsion as a calorie source was also studied in connection with the rate of amino acid utilization (Cannon *et al* 1954). These experiments show the dynamic nature of the body fat reserves and their capacity to contribute calories to the metabolic pool for a considerable period of time. Amino acids were used to some degree even with a subcaloric diet intake at least until the fat depots were exhausted. After exhaustion of fat depots amino acid utilization was markedly impaired and was corrected by supplying fat emulsion by injection. An important continuing contribution by this group has been the clear definition of the need for potassium to support amino acid utilization (Frazier *et al* 1956). Cannon *et al* (1953) and Rahman *et al* (1957) have further described the toxic effects of excess sodium in potassium depletion. These experiments support the point of view that excess sodium may lead to an intracellular displacement of potassium. In the last mentioned experiments growing rats were used. The repletion method was used throughout most of these studies however,

because of the speed and dramatic effects which were obtained by this technique

Evaluation of the protein quality of foodstuffs has not been a major aspect of the studies by the Chicago group. As pointed out by Cannon (1954) claims for assay value of the repletion method must obviously be supported by evidence that it possesses qualities of reliability, ease of performance and a reasonable quantitativeness. A number of reasons are presented why the principle of repletion is sound from the nutritional standpoint.

The Rutgers collaborative study (1950) showed good correlation between the repletion method and all other protein evaluation methods used. The method has, however, not been applied to a wide range of protein foods, nor has it been extensively studied in comparison with other protein evaluation methods.

### 5 *Repletion Method in Chicks and Pigs*

Protein assay in individual protein depleted chicks (Beatty *et al* 1957) provided the first application of the repletion method in this species. The basal diet consisting of cerelose, corn oil and purified vitamins, and salts, is comparable to that used effectively in rats. Chicks lost 25% of body weight in 6-16 days on nonprotein diet. The chicks were repleted for 2 weeks on diets made to contain 6.5, 9, or 12% protein. Some chicks were used for three assays. There appears no reason why this method should not be applied to small groups of chicks using standard equipment. Interestingly casein proved superior to lactalbumin for weight recovery in the chick. Also a 1:1 mixture of feather meal and lactalbumin performed better than either alone.

The repletion method as applied to baby pigs (Peo *et al* 1957), also appears highly reproducible. Pigs were used for three 7 day assays during their most rapid growth. Graded responses were reported for six levels of protein with greatest response to the highest level i.e. 22%. Frost and Sandy (1949) reported average responses for 5 rats ranging from 20 to 28 gm in ten consecutive repletion assays, a remarkable uniformity of response to a lactalbumin standard. Similar to the above work in pigs, greater resolution of differences and reproducibility of response came when protein feeding was high i.e. 0.24 gm nitrogen per day in rats and 22% protein in pigs. Two or four pigs were used in each assay in the Iowa work. Dried skim milk proved superior to two soybean meal diets in rate and efficiency of repletion. Effects on blood components were also studied (Peo *et al*, 1957). Although the repletion response was reflected in the albumin/globulin ratio blood constituent

measurements did not appear to offer advantages over weight gain as a criterion of protein repletion

## D CRITIQUE OF REPLETION METHODS

### 1 Depletion—Length of Depletion Period and Type of Diet

The method of assay of liquid protein hydrolyzates (Frost and Sandy, 1948a, b) differs in certain particulars from the method as originally developed and practiced by Cannon and his collaborators (Cannon *et al* 1944 Frazier *et al* 1947 Benditt *et al* 1947 Wissler *et al* 1948). One of these differences is the time and conditions of depletion. The latter work was first carried out (Cannon *et al* 1944 Wissler *et al* 1946) with a protein low diet (3E) based on natural materials including carrots brewers yeast and liver concentrate. In later studies (Frazier *et al* 1947 Benditt *et al* 1947) adult rats were depleted for approximately 3 months on the protein low diet (3E) and then transferred to protein free diet (4E) during the repletion periods. In still later studies (Benditt *et al* 1948a, b) the protein free diet has been used for the depletion and repletion periods. In the first instance (Frazier *et al* 1947), male rats (220–244 gm) were depleted on ration 3E about 3 months. Animals were then chosen for repletion on the basis of uniformity in initial weights percentages of weight loss (25–33%) and concentration of serum proteins and hemoglobin. In the later studies adult male rats weighing initially 288–350 gm were depleted of 15–25% of their body weight in a period of 5 weeks on the protein free diet.

Experience in this laboratory (Frost and Sandy 1948a) was that male rats (140–220 gm) lost 21–28% of body weight in 12 days on protein free diet. The original weights of the rats in this range did not appear to have any marked influence on the per cent weight loss during depletion or the magnitude of the repletion response. The 12 day depletion period appeared entirely adequate to elicit a uniform and maximum repletion response. More recently a method has been developed (Frost and Sandy 1949) for routine control purposes with an improved basal protein free diet wherein rats are used repeatedly with intermittent 7 day depletion periods between assays.

Optimal conditions of protein depletion for the purpose in question have as yet not been determined by direct experiment. One may assume that the object would be to thoroughly deplete the test animals of reserve protein without causing serious damage and without depleting to the point where edematous changes would mask true tissue weight loss. The following reports from the literature appear to have a fairly direct bearing on this question.



Addis *et al* (1936) showed that the liver is the primary organ to suffer a serious loss of protein, 40% being lost in a week's fasting. Kosterlitz (1947) states that rats fed protein free diet lose practically all labile liver cytoplasm in 4 days. Liver losses become much less rapid after the first few days, and thenceforth, according to Campbell and Kosterlitz (1946), follow an exponential curve as protein starvation proceeds. Thus, the methods (Kosterlitz and Campbell 1945; Campbell and Kosterlitz, 1948; Harrison and Lang 1945) based directly on regeneration of liver protein are of particularly short duration, allowing for only 2 to 7 days fasting or depletion on protein free diet.

The complete study of Wang and his co-workers (1949) is perhaps most pertinent to the question at hand. It is apparent from these studies that the major changes in the liver nitrogen occur within the first 12 days of protein depletion. From this time on subcutaneous edema appeared in a few of the animals, although the tendency toward edema was only slight. After about the 25th day of depletion the liver returns toward normal, both in chemical and cytologic character. The authors interpret the latter phenomena as representing an attempt within the animal body to conserve liver tissue after the first primary loss of more or less dispensable material. One may judge from these data that the depletion period on protein free diet would certainly not need to be more than 25 days, and that a period of 12 days may actually serve as well to accomplish the desired protein depletion.

## 2 *Methods of Feeding Nitrogen Supplements and Remainder of Diet*

A second question in the mechanics of the rat repletion method is the manner of feeding the protein supplement. The procedure of the Chicago group (Cannon *et al*, 1944; Cannon, 1945; Frazier *et al* 1947; 1949; Benditt *et al*, 1947; 1948a, b; Wissler *et al*, 1948) is as follows: the ration is so constructed that 1.35 gm of protein is obtained in 15 gm of ration, which supplies 48 calories. Consumption of the total allotment is reported to be complete for the better protein supplements, but is quite incomplete when an amino acid imbalance is presented (Wissler *et al*, 1947). The avidity for the ration is, indeed, a fair measure of the value of the amino acid mixture which is incorporated in the diet.

The protein supplement may be fed separately from the remainder of the diet as reported by Frost and Sandy (1949). This practice was an outgrowth of the separate feeding of liquid protein hydrolyzate and was found to work equally well for dry proteins. The protein supplements are weighed daily into cups which are then attached to the side walls of the cages in such a way that the adult rats have easy access to

them but cannot upset them. Daily protein feedings on the order of 0.12, 0.2, 0.24 and 0.36 gm nitrogen per day have been used. The level of 0.12 gm nitrogen per day is highly critical representing only about 1% of the total diet consumed.

Figure 3 illustrates the reproducibility of response to lactalbumin over eight 5 day assays in 5 rats. In practice in this laboratory rats are

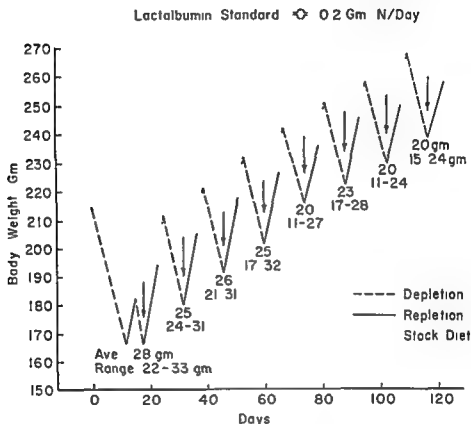


FIG. 3. The reproducibility of response to lactalbumin over eight 5 day assays in 5 rats using the rat repletion method.

generally not used for more than three or four repeat assays. Unless response is near maximal, undesirable variations develop between groups. It is desirable also where repletion rate is variable or poor to replete all groups 5 days prior to redepletion and assay.

The avidity for protein in general is very great and after a brief learning period the protein supplements are immediately and regularly consumed. The nonprotein diet is fed *ad libitum* as it is felt the appetite for the remainder of the diet should be in no way limited but should reflect the full effect of the supplement on the capacity for recovery. It is very clear that protein depleted rats will not take unbalanced amino

acid mixtures either added to the diet (Frazier *et al* 1947) or fed separately from other parts of the diet (Frost and Sandy, 1948a) In this type of diet then, in which all other components are presumed to be adequate, the most important appetite conditioning factor is the quality of the protein component For this reason it appears desirable to limit the feeding of the protein component at some desired critical level and to allow *ad libitum* intake of the bulk of the diet

Hegsted and Haffenreffer (1949) have provided experimental evidence that nutritional deficiency automatically limits appetite presumably by some mechanism related to total metabolism which can or cannot proceed at maximum rate, depending on the balance and supply of all nutritive factors One argument against paired feeding of the entire diets is that no pertinent information is gained over and above what is learned from experiments wherein *ad libitum* feeding, particularly of calories is practiced

*Ad libitum* feeding of both the protein and nonprotein components of the diet in the rat repletion assay was practiced in a few beginning experiments by Frost and Sandy (1948a) This type of experiment is applicable in assessing the general nutritive value of a protein and particularly the practical feeding value of a mixture of proteins The procedure of Cannon and his co workers, as described above supplies an abundance of calories The level of protein added to the basal diet is relatively low only 9% however, an intake of 15 gm daily would supply 1.35 gm of protein equivalent to about 0.2 gm of nitrogen The litter level is at least twice the minimum required for a half maximum rate of regeneration One may judge that in practice, this method allows essentially *ad libitum* consumption of calories, but a controlled feeding of the protein component The point at question is whether or not there is advantage in limiting the caloric intake at all by tying it to the protein intake

### 3 Effects of Different Feeding Levels of Nitrogen

Frost and Sandy (1948b) have studied the response to various controlled levels of nitrogen The dose response curve for a fibrin hydrolyzate was linear in the range 0.12–0.3 gm nitrogen per rat day Levels of 0.12 and 0.24 gm nitrogen per rat day were used (Frost and Sandy, 1949) in the assay of the Rutgers reference proteins and of beef blood fibrin and lactalbumin Table III shows the rat repletion response to these proteins at the two feeding levels together with the per cent of requirement supplied by the most limiting amino acid in each protein The fact that in each case at least one of the essential amino acids is supplied at a level below the maximum is sufficient for the failure of any

TABLE III  
RAT REFLECTION RESPONSES TO PROTEINS AT TWO NITROGEN LEVELS<sup>a</sup> LIMITING AMINO ACID DEFICIT CALCULATED FROM  
ESSENTIAL AMINO ACID CONTENT

Protein supplement	Repletion response			Limiting amino acid	% of requirement supplied
	at 0.12 gm	N/day gm	at 0.24 gm		
Egg albumin	38	± 3.5	80	Isoleucine	70
Whole egg (defatted)	33	± 1.4	66	Isoleucine	70
Beef muscle (defatted)	31	± 1.4	46	{Methionine Isoleucine}	44 47
Casein	31	± 2.9	45	{Methionine Tryptophan}	64 69
Peanut flour	8	± 6.3	32	Methionine	13
Wheat gluten	9	± 2.3	19	Lysine	20

<sup>a</sup> The milligram quantities of each of the essential amino acids supplied in an amount of protein containing 0.12 g N was calculated from the amino acid composition data for each protein (Frost and Sandy 1949). In each case as shown at least one of the essential amino acids was present at suboptimal level as compared with Cannon's minima (Steffe *et al.* 1950).

of these proteins to give a maximum response at this very low feeding level. It is obvious also, that at the 0.24 gm nitrogen level such proteins as egg albumin and whole egg will supply more than 100% of the requirement, but wheat gluten and peanut flour still fall far short. There is evidence that the methionine of casein is not well utilized (Frost and Sandy, 1948b) so that the apparent superiority of casein over beef muscle appears as an artifact. The differences in proteins fed at levels which supply more than the full requirements of all amino acids for maximum performance may be accounted for on the basis of amino acid balance, a subject discussed later in this paper.

#### 4 Use of Thyroprotein

Cabell and Earle (1951) reported that protein depletion could be hastened in rats by addition of 0.1-0.15% iodinated casein to the basal ration. It was their feeling that use of thyroprotein speeded up the repletion without effecting rate of repletion. The use of thyroprotein was applied first to the original method of Cannon, in which a nonpurified moderately low protein ration was used for slow (about 3 month) depletion. Cabell and Earle (1954) later used a purified nonprotein ration as described from our laboratory, but continued to use 0.1% Protamone during the much shorter 3 week depletion. The authors reported general correlation for the results of their repletion assay with the three other methods compared but failed to reach the desired precision. They nevertheless, point to many distinct advantages of the method and suggest that greater precision might be obtained at a higher level of nitrogen feeding than they used i.e., 0.1564 gm nitrogen per rat per day.

Considering the relative speed of the repletion method and the profound metabolic effects of thyroprotein one may question the advisability of its use to hasten depletion. Thyrotoxicosis is counteracted in part by dietary lipids particularly by bile acids and fats. Thus it would seem that more should be known about the effects of thyroprotein feeding before adapting this technique as part of the method.

As reported by Cabell and Earle (1954) consumption of the protein supplement was prompt and complete as offered at the same time each day. This corresponds with the experience in our laboratory (Frost and Sandy 1949), but again raises the question whether a single supplement during each 24 hour period provides optimum opportunity for maximum nutritive performance. It would be interesting to determine whether the same total supplement fed in two or more well divided doses each day would lead to greater weight recovery than when fed all at one time.

## IV METHODS OF CHEMICAL SCORING

## A DISCUSSION OF METHODS TO 1950

Evaluation of the nutritive value of a protein from knowledge of its amino acid composition still leaves much to be desired. Nevertheless it provides a real challenge and is capable of disclosing many useful correlations. It is particularly useful when considering the supplementary effects of mixtures of proteins or protein derivatives. The early work of Osborne and Mendel (1915) established the great improvement in the nutritive value of gliadin by the addition of lysine and of zein by the addition of both lysine and tryptophan. More recent findings have established many other limiting deficiencies in specific proteins such as the deficiencies of isoleucine and methionine in hemoglobin or isoleucine and tryptophan in blood albumin and of methionine in peanut and soybean.

Mitchell and Block (1946) and Block and Mitchell (1946-47) have tabulated the essential amino acid content of many of the common food proteins and have compared them with whole egg protein as a standard of excellence. According to their formulation the biological value of a protein ( $y$ ) may be roughly estimated from its maximum percentage deficit in an essential amino acid ( $x$ ) by the equation  $y = 102 - 0.634x$ . As pointed out by the authors, although correlation between the experimental and derived values are fairly good in general there are instances in which agreement fails. For instance the nutritive value of cereal proteins may be greatly impaired by heat although no change in the content of essential amino acids is apparent by analysis.

There are many other factors which operate against the clear application of the above method, particularly for comparisons between proteins of intermediate or high nutritive value: (1) the composition of whole egg protein cannot be assumed to be in the exact ratio of minimum requirements for each amino acid; (2) analytical values for each of the essential amino acids are not irrefutable; and (3) in a few instances whole egg protein has appeared somewhat inferior to certain other proteins.

One may also compare the composition of proteins with the established ratios of requirements as is done to some extent in Table III. The ratio of the essential amino acids of whole egg (Mitchell and Block 1946) has been calculated as a matter of interest and is as follows: tryptophan 1, phenylalanine 4.2, leucine 6.1, isoleucine 5.3, methionine 3.7, threonine 3.3, lysine 4.8, histidine 1.4, and valine 4.9. The correspondence of the above ratio to the ratios of requirements of rats and humans (Tables I and II) is probably no better nor worse than the correspondence between any of the other sets of ratios.

There is rather general agreement following the classic work of Rose that the essential amino acid composition of a protein provides the master key to protein quality. It is clear also that proteins of high nutritive value contain all of the essential amino acids in good amount and in ratios not greatly different one from the other. Analytical data (Frost and Sandy, 1949) on the Rutgers reference proteins showed that the essential amino acids accounted for about 60% of the total weight of the better proteins, but only about 40% of the poorer proteins. Both bio assay methods and methods of chemical scoring were easily capable of separating the better from the poorer proteins in this series as shown in Table III.

On the other hand the problem of differentiating the better proteins one from the other, on the basis of their essential amino acid composition (Frost and Sandy, 1949) as related to requirement is not clearcut, as seen in Table IV. Although peanut flour and wheat gluten display easily discernible limiting deficiencies, this is not equally true for defatted whole egg, egg albumin, casein and defatted beef muscle. The deficiency in casein is undoubtedly that of the sulfur amino acids, methionine plus cystine. Egg albumin appears to have slightly better composition to meet the needs of the protein depleted rat than does whole egg and this is borne out by the experimental results of Table III. Egg albumin is particularly rich in the sulfur amino acids, followed by whole egg, beef muscle, and casein.

In any event, none of the proteins appears to have a composition which closely matches the ratio of rat repletion requirements. Incidentally also the ratio of essential amino acids outlined for whole egg protein by Mitchell and Block (1946) varies considerably from that determined for defatted whole egg in this laboratory (Frost and Sandy, 1949).

The uncertainties in methods will undoubtedly become less in time and this coupled with the increasing knowledge of average species requirements may be expected to provide more exact means of estimating the ability of a protein or mixture of proteins to fulfill specific functions. Even with the attainment of this goal however, there will be continued dependence on animal assay methods if only because of their relative simplicity and directness.

## B DISCUSSION OF METHODS SINCE 1950

In recent years index to the nutritive value of proteins has been sought using various microorganisms. Growth rate of *Tetrahymena* *gelen* was used to evaluate protein hydrolyzates (Anderson and Williams 1951) and whole proteins (Pilcher and Williams 1954). Tern

TABLE IV

PROFILE OF RUTGERS REFERENCE PROTEINS AS COMPARED WITH THE REQUIREMENT FOR EACH AMINO ACID FOR A RAID RATE OF RITILENOV

Essential amino acid	Repletion minimum per 150 g rat/day	Per cent of requirement supplied by each protein at level of 120 mg N per rat day					
		Egg albumin %	Casein %	Defatted whole egg %	Peanut flour %	Wheat gluten %	Defatted beef muscle %
Tryptophan	14.5	97	69	97	77	69	83
Phenylalanine	45	111	90	98	71	82	64
Leucine	72.5	97	101	94	58	68	77
Isoleucine	61	70	70	70	43	52	47
Methionine	39	79	64	67	13	26	44
Threonine	43	84	79	88	42	44	79
Lysine	58	77	91	74	32	20	103
Histidine	21.5	93	116	75	73	71	126
Valine	51	114	108	116	53	55	71

The values underlined indicate the probable limiting amino acids and the severity of the limitations—the limitations in methionine cannot be interpreted clearly because of the complementary effect of cystine which is present in varying amounts in the different proteins (see text)

\* Values slightly revised from former published values (Cannon 1948 Steffee *et al* 1950)



*et al* (1956) attempted full evaluation of proteins by enzymatic liberation of amino acids, followed by microbiological assay and chromatographic analysis. Sheffner *et al* (1956) in somewhat different context determined the pattern of amino acids released by pepsin. This together with the amino acid pattern of the residue, was used to estimate the physiological availability of the essential amino acids. The values thus obtained for several proteins show remarkable agreement with biological values determined by Mitchell and Beadles (1950) in adult rats. There was, however, poor agreement for many proteins with the chemical score calculated by Oser (1951).

The fact that biological value of proteins is expressed as per cent has facilitated mathematical correlations between this method and the methods of chemical scoring. As stated elsewhere, the simple rat growth method is hard to beat for ease and reliability to test amino acid adequacy of proteins. Perhaps growth value of any protein can best be calculated as per cent of the response given by an isonitrogenous level of whole egg. Even more ideally the response can be in ratio to response to an optimum mixture of 12 amino acids. In a thorough analysis both the chemical score, such as Oser's (1951) (see Chapter 10) essential amino acid index, and bio assay should prove complementary. Combination of the two approaches should provide a clear picture of true feeding value, with indication as to which amino acids are limiting and to what degree.

To be fully used, amino acids must be liberated in the digestive tract at a rate permitting mutual supplementation. Thus, the rate of *in vitro* digestion of proteins by pancreatin appears to provide a useful index to *in vivo* availability (Ingram *et al*, 1949).

Lyman *et al* (1953) reported good correlation between solubility of cottonseed meal in 0.02 *N* sodium hydroxide and chick growth rate as measures of protein quality. This method accounted also for the gossypol content, an important factor limiting the nutritive value of cottonseed meal. More recent experiments (Green and Stephenson, 1955; Eagle and Davies, 1957) have not found nitrogen solubility of cotton seed meal in dilute alkali a reliable indicator of protein quality.

Methods of chemical scoring can be more and more widely applied with increasing knowledge of the essential amino acid content of foods. Williams (1955) and Lyman *et al* (1956) for instance list the essential amino acid content of a wide variety of feed ingredients.

### C LIMITING EFFECTS OF AMINO ACID IMBALANCE

The question of amino acid imbalance is large and complex and cannot be reviewed adequately here. That amino acid imbalance limits

nutrition and may even lead to severe disturbances in metabolism and toxicity is well known. Methods of chemical scoring deal thus far only with adequacy of each essential amino acid and do not attempt to predict the effects of overabundance of single amino acids or the effects of variations in proportions. There is sufficient variation in the diets of animals and groups of people and in the composition of individual proteins which have been used extensively in nutrition studies to warrant the idea that the body adjusts well to fairly large variations in the proportions of amino acids which are presented to it.

The extreme imbalance of certain proteins with regard to adequacy of the indispensable amino acids is heightened in some cases by the preponderance of certain amino acids which are actually inhibitory at high levels in the diet. This is notably true of gelatin and is related to the high glycine content of this protein as shown by Hier and his co-workers (1944).

Christensen and his co-workers (1948-1949) and Christensen and Streicher (1949) have presented new experimental evidence showing that the cells actually reject unbalanced amino acid mixtures. The studies show that the amino acid pattern of the cells can be upset by unbalanced amino acid levels in the extracellular fluid leading to a loss of amino acids from the interior of the cells. This provides a rational explanation as to why certain acute amino acid imbalances such as that reported for high levels of glycine or methionine work to the nutritional disadvantage of the diet. These phenomena are not within the present scope of chemical scoring.

#### D AMINO ACID IMBALANCE OF BLOOD

Previous studies demonstrating the isoleucine-methionine deficit in blood led Frost (1954) to test its repletion value. Diluted whole human blood rejected by depleted rats was readily accepted when fortified with isoleucine and methionine (Fig. 4). Subsequently Perdue *et al.* (1957) reported the amino acid composition of human blood and plasma as compared to dog blood. Amounts of L isoleucine and DL methionine needed to support rapid repletion were established for whole human blood. Human plasma unlike blood supported a slow rate of repletion but was also markedly improved by fortification with isoleucine and methionine. Dog and human blood were shown to differ considerably in content of isoleucine and methionine.

The unique leucine-isoleucine imbalance in blood plus the methionine deficit might be expected to lead to eventual nutritive failure in patients requiring repeated transfusions. The very slow entrance of the amino acids of transfused blood and plasma into body pools is recog-



The answer is undoubtedly direct assay in species for which such mixtures might be used economically. This is an example where again however the plan of mixtures for assay is best guided by complete knowledge of the amino acid patterns. Whether the amino acids are liberated in the digestive tract to allow mutual supplementation may also be indicated by *in vitro* digestion experiments. Most experiments with indirect methods of evaluation have been conducted to date with single proteins. The crux of their usefulness must come, however, in demonstrated ability to predict the value of practical mixtures.

## V SPECIFICITY OF AMINO ACID REQUIREMENTS

### A ESSENTIAL AMINO ACIDS

Rose defines an "essential amino acid" as one which cannot be synthesized by the animal organism out of materials ordinarily available at a speed commensurate with the demands for normal growth. This concept was based on rat growth studies extending over many years. A final classification based on this work was presented by Rose *et al* (1948). The classification for the requirements for maintenance of nitrogen balance in normal adult man as described by Rose (1949) differs in that histidine and arginine do not appear to be needed by adult man. The adult dog differs from adult man only in the requirement for histidine of Rose and Rice (1939). According to Frazier *et al* (1947) the adult rat requires the same 9 amino acids as the adult dog. The requirements of the chick for maximum rate of growth are more fastidious than that of either man or the rat according to Almquist and Grau (1944). The 8 key amino acids required by all species under all conditions are leucine, isoleucine, lysine, methionine, phenylalanine, threonine, tryptophan, and valine. In addition to these the chick requires for maximum growth histidine, arginine, glycine, and glutamic acid.

### II THE ROLE OF NONESSENTIAL AMINO ACIDS AND OTHER SOURCES OF NITROGEN

The fact that glutamic acid and proline like arginine will stimulate growth rate in weaning rats fed a mixture of the 10 amino acids was indicated by the work of Womack and Rose (1947). Later Rose *et al* (1948) showed that removal of glutamic acid from a mixture of 19 amino acids resulted in only slight inhibition of growth rate which was statistically of doubtful significance. Still more recently Rose and his co-workers (1949) reported the ability of diammonium citrate, glutamic acid, glycine, or urea to provide the extra nitrogen in addition to the

10 essential amino acid mixture, required for synthesis of the nonessential amino acids

The work of Lardy and Feldott (1949) is very clearcut, even though limited to only a few rats in showing the complete dispensability of all but 10 amino acids for the growing rat. In these studies, an isonitrogenous amount of diammonium citrate was used to effectively replace the 8 nonessential amino acids of the complete amino acid mixture (Mixture XXIII) of Rose *et al* (1948).

Work in this laboratory by the method of liquid supplement feeding to adult protein depleted rats confirmed the above reports as to the ability of diammonium citrate to replace the nonessential amino acids. Unlike the young rat the adult protein depleted rat requires only 9 amino acids responding maximally to isonitrogenous supplements of arginine or glutamic acid or diammonium citrate. In agreement with the findings of Rose *et al* (1949) for the growing rat our findings in the adult protein depleted rat place glycine in a position of intermediate effectiveness and urea least efficient of all. Rose *et al* (1949) reported that ammonium acetate was fully as effective as diammonium citrate, thus ruling out involvement of the citrate ion.

In our studies (Frost and Sandy, 1951), the mixture of 9 essential amino acids in the proportions used by Steffee *et al* (1950) was fed at a level to supply exactly twice the minimum amounts of each amino acid required to support a maximum rate of repletion. This is still a very low level of nitrogen amounting to only 138 mg nitrogen per rat day, of which 36 mg was  $\beta$  amino acid nitrogen of the unavailable isomers of isoleucine, threonine, and valine. Thus the amount of nitrogen physiologically available for repletion in these studies was only about 100 mg per rat day, well below the level required for maximum rate of repletion. Despite this critical feeding level of total nitrogen there was a fair response to the minima mixture alone. However when about one third of the total nitrogen of the minima mixture was replaced by diammonium citrate, arginine or glutamic acid the rate of weight gain was increased more than 50%. One third isonitrogenous replacement with urea gave about a 25% weight increase over the minima mixture alone. Thus it would appear that the ability of rats to convert the essential to nonessential amino acids is rather poor and that the other sources of nitrogen mentioned are much more readily used by the body for this purpose. Results of replacement of part of the essential amino acid nitrogen by glutamic acid, arginine, urea or diammonium citrate are shown in Table V.

Foster *et al* (1939) had clearly demonstrated that dietary ammonium  $N^{15}$  is rapidly incorporated into rat tissue proteins. The above findings

establish the utility of ammonia nitrogen in an entirely different way. In his general review of the role of the isotopes to reveal the dynamic state of body constituents Schoenheimer (1942) reported the virtually complete excretion of dietary urea  $N^{15}$  in unchanged form. It is revealing then from the above work that under conditions of stress, the body can actually use urea to some extent as a source of nitrogen and can use urea nitrogen even more efficiently than essential amino acid nitro-

TABLE V

RESPONSE OF ADULT PROTEIN DEPLETED RATS TO THE 9 ESSENTIAL AMINO ACID MINIMA MIXTURE FED AT TWICE THE MINIMUM LEVELS: EFFECT OF REPLACING PART OF THE ESSENTIAL AMINO ACID N BY VARIOUS NONSPECIFIC NITROGEN SOURCES

Mixture fed	Av. 12 day weight gain gm	Standard error gm
Minima mixture alone (1st assay)	19	
Minima mixture (Repeat assay in same rats)	22	$\pm 2.2$
Minima with 19.3% arginine N (1st assay)	34	
Minima with 19.3% arginine N (repeat assay)	32	$\pm 2.0$
Minima mixture alone	21	$\pm 2.6$
Minima with 32.4% urea N	26	$\pm 2.4$
Minima with 32.4% arginine N	35	$\pm 2.3$
Minima with 32.4% glutamic acid N	36	$\pm 1.9$
Minima mixture alone	23.2	$\pm 1.9$
Minima with 10% ammonium acetate N	34	$\pm 3.5$
Minima with 20% ammonium acetate N	36	$\pm 4.3$
Minima with 30% ammonium acetate N	43	$\pm 8.4$

Four sets of experiments are shown. Groups of 5 to 11 rats were used in each experiment. All solutions were made to contain 0.55 to 0.65% nitrogen. Standard nitrogen allotments of 0.138 gm N per rat day in 25 ml were fed throughout. This level of nitrogen provided twice the amounts of Cannon's minima for each of the essential amino acids in the case of the minima mixture alone. Replacement of part of the nitrogen of the minima mixture by other nitrogen sources was made as shown.

gen for general synthetic purposes. In the adult protein depleted rat as in the growing rat there is a wide range of nitrogen compounds other than the nonessential amino acids which would need to be synthesized from dietary nitrogen. These compounds must all be synthesized physiologically from ammonia and carbon residues derived from normal metabolism cycles.

It is well established that two chief reactions occur in the tissues with regard to changes in the nitrogen moiety (1) transamination and (2) oxidative deamination and subsequent urea formation. It is also

fairly well established that alanine and glutamic and aspartic acids enter most readily into transamination reactions with the  $\alpha$  keto acids known to occur in the body. Glutamic acid is an efficient source of nitrogen to replace all other nonessential amino acids, and by the same token alanine and aspartic acid serve well in the same capacity. On the other hand none of the essential amino acids enter readily into this reaction and, therefore, must be degraded by decarboxylation or by oxidative deamination. There is evidence that the latter reaction occurs in the liver and leads directly to urea formation. In the body economy one might expect that reaction mechanisms would be in the direction of conserving the essential amino acids, rather than degrading them rapidly and this indeed, appears to be the case.

The beauty of the isotope technique to trace the metabolic fate of amino acid nitrogen is exemplified in studies by Wu and Rittenberg (1949) in which the metabolic fate of L aspartic acid is described. The findings suggest that aspartic acid is so rapidly deaminated that its amino group behaves metabolically like ammonia. In regard to the isotope studies it may be worthy of note that the first broad interpretation was that all L amino acids are readily deaminated. This idea is apparent in the extensive review of the role of the dicarboxylic acids in nitrogen metabolism by Braunstein (1947). On the contrary the nutritional studies in this laboratory with protein depleted rats clearly support the idea that the deamination of essential amino acids is quite limited. One deals both with equilibria and rates of reaction and coupled with these factors in utilization from the nutritional viewpoint is the factor of renal excretion. For example normal animals on normal diets excrete urea almost quantitatively whereas animals receiving only the essential amino acids use urea to fairly good advantage. Furthermore in balance studies these animals did not excrete much more urea than did rats receiving only the 9 essentials. Rats receiving one third of their total nitrogen intake in the form of diammonium citrate did not excrete appreciably more ammonia and urea nitrogen than rats on normal diets.

### C THE ROLE OF ARGININE

The situation with regard to arginine requires special consideration. As first reported by Frazier *et al* (1947), rats receiving only 9 essential amino acids did as well as animals receiving a 16 amino acid mixture patterned after casein or the 10 amino acid mixture containing arginine. This is contrary to our experience as we have consistently found that almost any source of other than essential amino acid nitrogen will serve to improve the response over that shown by the mixture of 9 essentials.

alone. Following the work of Frazier *et al* (1947) it was noted in the joint work of Wissler *et al* (1948) that the addition of arginine to a 9 amino acid mixture did stimulate appetite in normal adult animals.

A possible explanation of the failure of Frazier *et al* (1947) to show an effect of the nonessential amino acids over and above that of the 9 essentials is found in the comparative amino acid mixtures used by these authors. The mixture of 16 amino acids patterned after casein contains a rather low level of methionine and only a trace of cystine. One would expect the sulfur amino acids to be limiting as in casein. In the comparative feeding experiments however the rats received a much higher level of methionine from the 9 than from the 16 amino acid mixture. Thus the nutritional advantage related to higher methionine level in the mixture of only the essentials may have balanced the nutritional advantage which we would ascribe to the presence of the nonessential amino acids in the 16 amino acid mixture.

The role of arginine in the nutrition of the growing rat had been a subject of continued study at the University of Illinois for many years. The results of comprehensive studies of Borman *et al* (1946) confirmed the earlier evidence as to the requirement of arginine for maximum growth in the rat. The diet used was much improved with regard to purity and adequacy of vitamin supplements over diets formerly available so that average growth rates were greatly increased and the effect of arginine was clearcut. Finally Womack and Rose (1947) demonstrated the fact that either glutamic acid or proline could partly substitute for an isonitrogenous addition of arginine. The findings were interpreted "as evidence that the three amino acids are mutually interconvertible in the organism of the rat but at different rates as exemplified by their different influence upon growth. In view of our recent findings in the adult rat one would say that glutamic acid and arginine, at least are readily used as sources of ammonia nitrogen for conversion to all other nonessential amino acids. Emphasis was placed in the report of Womack and Rose (1947) on the essentiality of arginine and the relation of glutamic acid and proline to arginine. The requirement of the growing rat for arginine appears to be well established from the work of Rose and his collaborators. It would be of interest in the light of recent work to determine whether or not there is a sparing effect of fairly high levels of glutamic acid and diammonium citrate for arginine in the growing rat.

#### D THE ROLE OF GLUTAMIC ACID

Concluding experiments of Rose *et al* (1948) showed that in 28 days rats receiving only 10 amino acids gained about 70 to 75% as much as



litter mates which received 19 amino acids. Although addition of glutamic acid to the 10 amino acid mixture stimulated growth, removal of the glutamic acid from the 19 amino acid mixture did not have a statistically significant effect. The authors stated further that under similar conditions the influence, if any, of glutamic acid upon growth is much less than that manifested by arginine. In view of these facts glutamic acid was classified as a dispensable dietary component for the rat and this conclusion is well supported by the work cited from other laboratories.

Establishment of the complete dispensability of glutamic and aspartic acids for the nutrition of the rat is of interest with regard to the preparation of intravenous amino acid mixtures, wherein the presence of a high proportion of these amino acids is undesirable.

#### E RATIO OF ESSENTIAL AMINO ACID NITROGEN TO NONSPECIFIC NITROGEN

On the basis of experiments in this laboratory (Frost and Sandy, 1950) it would seem that the ratio of essential to other than essential amino acid nitrogen is of primary importance when studying the efficiency of any given source of other than essential amino acid nitrogen. Although the optimum level of other than essential amino acid nitrogen has not been determined accurately it is clear that one third substitution of the total nitrogen in the form of glutamic acid, arginine or diammonium citrate supports an excellent response. The effect of these sources of nitrogen is pronounced at the above level. Very significant effects were noted also for arginine, glycine and a mixture of glycine and alanine at one fifth of the total nitrogen. Thus, it is clear that small additions or substitutions of various sources of nitrogen to the essential amino acid mixture would all be expected to produce a supplementary sparing effect, up to an undetermined optimum ratio.

The conditions used by different laboratories in showing the utilization of sources of other than essential amino acid nitrogen are all quite different and are, therefore, difficult to compare. Rose *et al* (1949) measured the stimulation of growth of young rats when other sources of nitrogen were added to the mixture of the 10 essential amino acids fed at the predetermined minimum levels, i.e., 8.82% of the diet. Under these conditions of additive effect, glutamic acid and diammonium citrate produced the greatest growth, glycine was next, and urea poorest. In another type of experiment, these authors fed only one half the minimum levels of the 10 essentials to 3 young rats and measured the effects of additions on the maintenance of weight and nitrogen equilibrium. Only the L amino acid forms were used in this experiment, and the

mixture comprised only 3.46% of the diet. Supplements of urea or diammonium citrate were made to supply an amount of nitrogen equal to that of the amino acid mixture. Under these conditions both supplements induced strong nitrogen retention accompanied by some weight increase.

Lardy and Feldott (1949) compared weight gain and nitrogen retention of young rats on (a) the 18 amino acid mixture (Mixture XXIII) of Rose *et al.* (1948) at 10.3% of the diet, (b) the 10 essential amino acid mixture at 8% of the diet, and (c) the 10 essential amino acid mixture at 8% and diammonium citrate (2.15%) to equal the non-essential amino acid nitrogen of the 18 amino acid mixture. Mixtures of the 10 L amino acids and the same plus diammonium citrate were also studied. The level of essential physiologically active amino acids in the diets was 11.4%. The data presented showed that the diammonium citrate replaced all of the nonessential amino acids in stimulating growth rate above that shown with the 10 amino acids alone. The balance data further showed that urinary ammonia nitrogen loss following diammonium citrate feeding was not far greater than that following amino acids alone. With regard to the ratios of the different forms of nitrogen it was of interest to calculate that 19% of total nitrogen of the mixture plus diammonium citrate consisted of  $\text{NH}_3 - \text{N}$  and 30.7% of total nitrogen was present as  $\text{NH}_3 - \text{N}$  plus arginine nitrogen.

The experiments in this laboratory with adult protein depleted rats as compared with the experiments with growing rats, involved controlled rather than *ad libitum* amino acid feeding. The essential amino acids were offered at twice the levels required for rapid recovery. Substitution of a part of the nitrogen of the essentials by various other sources of nitrogen elicited a much greater response than that given by the essential amino acids alone. Thus it became clear that the essential amino acids themselves are not readily available as sources of nitrogen for conversion to other nitrogen components of the body. The conversion of methionine to cystine and of phenylalanine to tyrosine appears as an exception to this generalization. This failure in ready conversion of the essential amino acids to other general forms of nitrogen may represent a real difference in metabolism of the two classifications. There is of course good reason for the body to conserve essential amino acid nitrogen if one wishes to invoke purely teleological reasoning. It is nevertheless, surprising that the body can utilize urea even more efficiently as a non-specific nitrogen source than it can a complete mixture of the essential amino acids.

## VI RACEMIC AMINO ACID MIXTURES

A major problem in both the experimental and developmental use of mixtures of synthetic amino acids comes in the fact that the unnatural forms of certain of the essential amino acids are not utilized. Unfortunately this is true for certain of the most expensive and rare amino acids namely, threonine, isoleucine and valine.

Tryptophan presents a special problem in that the DL forms are both used by the rat and the dog although the D form may be less well utilized than the L form. Only L-tryptophan is used by the human according to Rose (1949) and Heber and Berg (1949). Thus, assays of protein preparations with rats may not give a clear prediction of the value for human use with regard to the content of this amino acid. This circumstance deserves special consideration because of the relative instability of tryptophan to chemical treatments such as acid hydrolysis.

Rose (1949, 1938) has discussed the activity of the D and L forms of each of the essential amino acids for the rat and the human. A review of the literature pertinent to the activity of the optical isomers of each of the amino acids for various species will not be attempted here (see Chapter 4 by Berg). It would appear, however, from the results to date that the only significant discriminatory difference in utilization by rat and man is found in the case of tryptophan, as discussed above. Neither species according to Rose can utilize the D isomers of valine, leucine, isoleucine, threonine and lysine. Both species can use racemic methionine fully and can make partial inversion of D-phenylalanine.

### A THE QUESTION OF INHIBITION BY D AMINO ACIDS

One might expect that the unusable isomers of the amino acids would show some interference in the normal metabolism of their usable enantiomorphs. That this is indeed true, at least in the case of D-valine and D-leucine, has been shown by Fling and Fox (1945) in experiments with bacteria. D-Alanine was not inhibitory, and this was explained theoretically on the basis that the methyl group of alanine is sufficiently small that it would not intervene or cause steric hindrance as is thought to be the case for the larger side chains of leucine and valine. The antipodal specificity of proteolytic enzymes is well known and it would not be surprising if the inhibition caused by certain of the D-amino acids comes at that point. The further illuminating finding has been reported by Prescott *et al.* (1949) that DL-tryptophan is not as effective in meeting the growth requirements of certain microorganisms as a one-half level of L-tryptophan. The presence of D-tryptophan in this case made for an increased requirement for L-tryptophan—a situation common to many reversible competitive inhibitions.

Evidence for the inhibitory effect of D amino acids in animals is less clear than that for bacteria; however the preponderance of evidence indicates that competitive inhibition does take place. Wretling (1948) reported that a mixture of 10 DL amino acids patterned after Rose's minimum requirements for the rat gave a greater growth response at a level of 20% than at 30 or 40% in a purified diet. The results were interpreted as meaning that completely racemic substances contain toxic substances. Because Wretling did not supply any source of other than essential amino acid nitrogen in the diet, there remained the possibility that the inhibition was caused by an overloading of the capacity of the body to convert essential amino acid nitrogen to nonessential amino acids and other nitrogenous compounds necessary for growth.

The presence of racemic valine, isoleucine, threonine and phenyl alanine in three of the mixtures studied by Lardy and Feldott (1949) did not appear from their data to inhibit intake or weight response as compared with mixtures of only L amino acids. On the other hand Brand and Bosshardt (1948) reported that a mixture of L amino acids duplicating the composition of  $\beta$  lactoglobulin supported the same growth in mice as a corresponding amount of the whole protein, whereas use of certain DL amino acids in the mixture impaired the growth response. It has been repeatedly observed in this laboratory in the aforementioned rat repletion studies that amino acid mixtures containing the racemic forms of valine, isoleucine and threonine are accepted with much less avidity than are complete hydrolyzates of fibrin supplemented only with DL tryptophan. This is true even when adequate sources of other than essential amino acid nitrogen are supplied. A very striking difference is the slow rate at which the rats consume the amino acid solutions compared with the eager and rapid drinking of hydrolyzate solutions. The crucial experiment to finally answer this point must await the preparation of a mixture of the 10 essential L amino acids in fair quantity. The suggestion is clear, however, that certain of the D-amino acids exert inhibitory effects on metabolism in various forms of life.

Albanese (1949) has recently presented the thesis based on certain indirect evidence in work with human infants that DL tryptophan may have some untoward effects not shown by L tryptophan when used as supplements to tryptophan low diets. There is need for a critical and fundamental study in humans of the effects of DL tryptophan as compared with L tryptophan. The addition of DL tryptophan to parenteral amino acid solutions is now common practice and no gross aberrant effects have been noted. The question is a complex one, however, and may require more basic study than it has received.

In comparing rat growth on mixtures of amino acids with casein and

casein hydrolyzates, Ramasarma *et al* (1949) noted slower growth on the former. One possible explanation which they advanced for the slower growth on the amino acid mixtures was the presence of D amino acids. As stated at the levels used, they may not produce any symptoms of toxicity but may cause a slight depression of growth.

A paper by Van Pilsum and Berg (1950) bears directly on the effects of the D and L forms of amino acids on growth. The authors reported that the L forms of the 10 essential amino acids as components of a DL mixture constituting 22.4% of the diet grew less well than control rats fed only the L isomers at a dietary level of 11.2%. However, when only half as much DL phenylalanine, tryptophan, methionine, and arginine and an intermediate level of DL histidine were included the resulting 18.6% of DL amino acids promoted as good growth as that attained on the L mixture.

The interesting finding was then made that removal of half of the DL methionine overcame the growth retarding effect of the 22.4% DL mixture. Comparative tests showed that the growth retardation produced by the natural L isomer of methionine was greater than that produced by either the DL or the D modification. The toxicity of excess methionine is well known. Both forms of methionine can be used and it is possible that an excess of L methionine exerts more toxicity than the D form. This situation of course may not apply for those amino acids for which the D amino acid is not used.

Van Pilsum and Berg concluded that contrary to the often repeated conjecture that the D forms of the essential amino acids may be toxic in the rat, proportionately large amounts can actually be fed as components of DL amino acid mixtures without producing any apparent growth retarding or other deleterious effect. Although it is clear from these experiments that the D amino acids are for the most part inert, certain questions require investigation before the argument can be entirely closed regarding their reputed inhibitory effects. It will be noted that in the above cited work, growth rates were slower on both the L and DL amino acid mixtures than on properly fortified casein hydrolyzates.

An important consideration in interpretation of the work of Van Pilsum and Berg is the absence of nonspecific nitrogen from their amino acid mixtures. Although these authors added ammonium citrate and glycine to their essential amino acid mixtures in a few studies, these additions did not appear to have any effect on growth, and the question was not examined further. The absence of a sufficient concentration of other than essential amino acid nitrogen would however be expected to inhibit response to a measurable degree judging from the work of Lardy and Feldott (1949) as well as the work in this laboratory. The possi-

bility appears therefore that there may have been a second variable at work other than the optical form of the amino acid groups. Judging from all of the evidence it would seem desirable to have present in such a study an adequate source of other than essential amino acid nitrogen so that synthesis of nonessential nitrogen compounds could proceed more readily than is the case when only essential amino acids are present. The excellent study of Van Pilsum and Berg points up the need for this additional experimental approach to the effect of D-amino acids.

## VII RECENT STUDIES WITH L AMINO ACID MIXTURES

Reichel *et al* (1957) studied the value of single nonessential L amino acids diammonium citrate urea or biuret to promote growth in weanling rats when added to a mixture of essential L amino acids patterned after the rat carcass. L Glutamic acid was the most effective single supplement for growth feed conversion and net nitrogen utilization followed by L alanine L aspartic acid L asparagine L proline L glutamine diammonium citrate urea biuret glycine and L serine. In addition to measuring growth rate the rapid procedure of Miller and Bender (1955) and Bender (1956) was used for estimating net utilization of these nonspecific nitrogen sources. Both measurements placed the nitrogen supplements in the same relative order of efficacy. Interestingly, in this study an excess of the entire group of essential L amino acids proved almost as effective as L glutamic acid when judged by growth food efficiency and net nitrogen utilization. This finding indicates that the presence of several DL amino acids in the essential amino acid mixture may have accounted for their poor repletion response as compared to nonspecific nitrogen sources.

The above results together with those of Greenstein *et al* (1957) provide final answers to many questions which complicated the earlier use of DL mixtures in rats. As stated by the latter, "Animal tissues are geared to handle metabolically the natural L forms of the amino acids, and although some of the D forms can be more or less rapidly inverted (Berg 1953), they may in mixtures interfere in each other's inversion. This has been clearly shown in the experiments of Wretling (1956) and the peculiar behavior of D valine in the presence of the D-isomers of other amino acids such as leucine and isoleucine (Womack *et al* 1957), gives rise to the typically confusing situation evoked by the presence of appreciable amounts of unnatural materials in an experimental diet. It should hardly be necessary to press this point but the substitution of financial economy for quality of experiment has little to recommend it."

Although practical application is still far off the time may come

when essential L amino acids can be used economically for intravenous feeding. L Glutamic acid and L aspartic acid are poorly tolerated intravenously. Thus the search may well go on to establish a suitable low cost source of nonspecific nitrogen to supplement the 10 essentials (counting histidine and arginine). Whether L tryptophan can replace DL tryptophan to advantage in *in vivo* protein hydrolyzates should also be determined.

Birnbaum *et al* (1957) suggest that, No amino acid is nonessential for maximum growth. The capacity of the body to build purines, pyrimidines, porphyrins, etc. is clearly dependent on adequate supplies of metabolizable nitrogen. Just how well simple combination of nonessential L amino acids with ammonia sources can fill specific needs has yet to be determined. Work in intermediary metabolism has shown many common pathways for interconversion between amino acids, carbohydrates, and lipids. One can safely generalize that hormonal control of metabolic patterns differs greatly between individuals and particularly in those who are ill. Thus it may come to pass that most of the tissue amino acids do have nutritional import. Use of massive doses of glutamic acid in mental disease states and the use of arginine (Fahey *et al* 1958) to reduce ammonia intoxication are examples.

Conversely, Frost and Sandy (1951) concluded on the basis of their studies that about 20-30% of the nitrogen, supplied with an amino acid mixture, should be other than essential amino acid nitrogen for maximum repletion. This is much less nitrogen in the form of nonessential amino acid nitrogen than is present in proteins of the highest biological value. The question still appears to remain open as to whether a man-made mixture of L amino acids will actually surpass the highest biological value proteins in nitrogen efficiency. It is quite conceivable and of some import, particularly for intravenous feeding, that 9 or 10 essential L amino acids supported by other easily available sources of nitrogen may yet be shown to be the most efficient source of nitrogen. A revealing experiment now will be to test solutions of the 9 essential L amino acids fortified with arginine and an ammonium salt. Avidity for such mixtures, as compared to that for protein hydrolyzates, will indicate whether such simple mixtures supply the full physiological need. Fahey *et al* (1958) have clearly shown the value of arginine, ornithine, or citrulline to prevent ammonia intoxication in dogs receiving a mixture intravenously of the 8 essential L amino acids plus L alanine as a non-specific source of nitrogen. Final proof of the over-all value of such mixtures given intravenously is a worthy objective.

## VIII AMINO ACID ANALOGS

The DL hydroxy analog of methionine represents thus far the only commercial application for hydroxy or keto analogs of amino acids. Hydroxy DL methionine substitutes equally for methionine in chick rations (Bird 1952 Michlin and Gordon 1957). When a low level of soy protein was fed methionine proved superior to methionine hydroxy analog as a supplement to promote growth (Sullivan and Bird 1957). When a nonspecific source of nitrogen was also supplied growth response for methionine hydroxy analog equaled that for methionine addition. This demonstrated utilization of nonprotein nitrogen by the chick presumably for amination of the hydroxy acid.

Gordon and Sizer (1955) found DL methionine less efficient than equimolar supplements of L methionine D methionine or methionine hydroxy analog (calcium DL 2 hydroxy 4 methylthiobutyrate) for chick growth. Resolution of optical isomers is often an expensive and imponderable one for the chemist. The body may expedite this job given the proper metabolic fragments. Thus it is conceivable that appropriate mixtures of the hydroxy or keto analogs of many or all of the essential amino acids with adequate sources of nonspecific nitrogen sources may in time prove advantageous for parenteral feeding.

Rose *et al* (1949) made the significant disclosure that the  $\alpha$  keto analogs of valine and isoleucine promote excellent growth in rats as the sole sources of these amino acids. The suggestion is made that they undergo asymmetric amination *in vivo* to supply the corresponding L amino acids. If the  $\alpha$  keto analogs are less costly to synthesize strategic use might be made of them together with a source of ammonia in idealized mixtures.

## IX SUMMARY

There is an obvious need for general acceptance official or otherwise of methods to measure nutritive value of proteins. Such methods should be simple and low cost for wide application. Requirements for maximum performance should be known in terms of each amino acid ultimately the L amino acids. Requirements for nonspecific sources of nitrogen to support maximum use of the essential L amino acids should likewise be established. The growth and repletion methods satisfy many of these criteria. Amino acid requirements as presently established are quite similar. Both methods clearly measure the adequacy, balance and availability of the amino acid composition of proteins for tissue synthesis. Careful comparisons are needed to establish their relative reliability and convenience for specific applications.

Determination of biological value has been precisely studied but has



obvious limitations for broad applicability. It is nevertheless the method of choice to measure maintenance and reproduction requirements in adults. The nitrogen balance technique can pass final judgment also as to utilization of protein hydrolyzates on injection.

These methods, properly standardized and extended to various species appear to provide a reasonable base for broad evaluation and comparison of amino acid mixtures, individual proteins, protein hydrolyzates, and protein foods.

The growth and repletion methods may be supplemented by calculation of protein efficiency ratio, or, going further, net protein utilization. There is considerable doubt, however, whether these refinements confer precision over the basic methods alone. In any case, final definition in terms of L amino acids appears so close at hand as to preclude speculation on these points.

Space has not permitted full consideration of all of the literature and methods which deserve attention. The rat repletion method is most extensively discussed. Although highly sensitive to amino acid adequacy and balance, this method does not fully predict the value of partial protein hydrolyzates given intravenously. It serves well as a production control method due to its rapidity and low cost.

Methods of chemical scoring provide theoretical support for bio assay, but are now no less complicated or less costly than bio assay. The same holds true for amino acid liberation from intact proteins by enzymes. Thus far direct assay of protein value by microorganisms appears to offer inadequate correlation with animal assay.

Various nonspecific forms of nitrogen support anabolism in presence of the essential amino acids. The question remains whether ammonium salt can effectively replace all or part of the nonessential amino acids. Most studies are clearly complicated by the use of DL amino acid mixtures. The alpha hydroxy or keto analogs of some amino acids may avoid this complication. These basic questions will be resolved only when sufficient L amino acids and suitable analogs are available for precise study. By the same token amino acid requirements require re evaluation in terms of only the L forms.

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## CHAPTER 10

# An Integrated Essential Amino Acid Index for Predicting the Biological Value of Proteins

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## I INTRODUCTION

The utilization of dietary proteins for growth and maintenance is dependent on the presence and relative amounts of the essential amino acids and on the degree and rate of digestion. The solubility and susceptibility to enzymatic attack of proteins are determined by their structural organization as well as by the presence of protective cellular tissue especially in foods of plant origin. The coefficient of digestibility of protein in foods is in general uniformly high. However the biological value of a protein or proportion of absorbed nitrogen retained for utilization by the animal organism varies to a considerably greater degree. The biological value might by analogy be called the coefficient of utilization. It is a function of the available (i.e. absorbed) content of essential amino acids the amino acids which the animal cannot synthesize at a rate commensurate with the demands for normal growth (Rose 1938). In the complete absence of any of the amino acids indispensable for an animal species normal maintenance growth and survival are not possible. The overall adequacy of a diet with respect to protein depends upon the extent to which the component proteins complement each other in filling the essential amino acid needs of the individual and collectively furnish enough of the amino acids nonessential as well as essential to replace catabolic losses and to satisfy anabolic requirements.

Since the advent of modern microbiological and chromatographic methods for the assay of amino acids it has become relatively simple to determine all of the essential amino acids. Instead of focusing atten-

tion on the one or two limiting acids in proteins as a basis for their nutritional assessment, it has become possible to consider the content of all of the essential amino acids as an integrated whole

## II EMPIRICAL RATINGS OF PROTEINS BASED ON ESSENTIAL AMINO ACID CONTENT

Mitchell and Block (1946) devised a system of chemical scores based on the amount of the essential amino acid in greatest deficit in a protein compared to the level present in a reference protein selected for its nutritional excellence. Despite the empirical nature of this system, the chemical scores were shown (Mitchell, 1951a) to have a high degree of correlation ( $r = +0.95$ ) with published biological values for a series of proteins. However, as Mitchell has pointed out in his excellent reviews (Mitchell, 1954a, b) on the dependence of biological values of food proteins on their content of essential amino acids the chemical score is an index of the value of protein for growth only since it assumes that the absence of an essential amino acid renders the protein completely unavailable even for tissue maintenance, an assumption not consistent with reported observations (McCallum 1911, Mason and Palmer 1935).

Each essential amino acid may be considered as the keystone in an arch, without which a stable protein structure cannot be built. It would seem more reasonable that the rating of a protein for nutritional quality should take into account its entire contribution of essential amino acids not merely the one in greatest deficit with respect to a nutritional standard. Hence the writer was intrigued with the possibility of devising a means of representing the entire essential amino acid spectrum of a protein by an integer which might be correlated with experimentally determined coefficients of utilization, such as the biological value of Mitchell or the nitrogen balance index of Allison (Allison and Anderson 1945).

Studies in protein utilization have directed attention to the importance of the time factor in absorption. For example it was shown that too long delay in supplementation of an incomplete amino acid mixture with the missing factor failed to improve retention (Berg and Rose, 1929; Elman 1939; Henry and Kon, 1946; Geiger 1947). This led to the working hypothesis that "for optimum utilization of food proteins all essential amino acids must not only be available for absorption but must also be liberated during digestion *in vivo* at rates permitting mutual supplementation" (Melnick *et al.* 1946). In other words for most efficient construction of the protein arch every essential building block must be available at the site and at the time of synthesis.

The probability of two or more events occurring simultaneously is a

function of the product of the probability of their individual occurrences. It follows therefore that the probability that all of the amino acid building blocks will be available at the site of synthesis (that is to say for biological utilization) is a function of their product and not their sum. Kuhnau (1949) proposed a method of rating proteins according to the sum of the percentages of essential amino acids. A protein completely lacking one of the essential amino acids and hence incapable of supporting growth should theoretically at least be assigned a rating of zero. An index based on the product of these percentages instead of their sum would yield the theoretical value of zero in the case cited.

However even the most deficient proteins like gelatin or gluten yield low but not zero biological values under experimental conditions. This probably arises from the fact that the nitrogen balance procedure involves the use of short test periods during which amino acids lacking in the test proteins may be picked up from intestinal debris or from the products of tissue catabolism albeit in trace amounts but sufficient to permit some utilization of incomplete dietary proteins. Hence in order to correlate observed biological values with an integrated essential amino acid index it is necessary to assume the presence of a small content of even the missing essential amino acids.

### III THE STANDARD OF REFERENCE

The nutritional value of any protein or mixture of essential amino acids for an animal must be related to its individual needs for growth and maintenance. The age, sex and status of an animal with respect to pregnancy, lactation, disease, convalescence, nutritional history and so on are significant factors in the determination of individual requirements. In view of the many variables it is obviously invalid to make the general assumption that the amino acid requirement of a specific segment of the population can serve as a universal yardstick for the evaluation of dietary proteins. For this reason it seemed preferable to adopt as the standard a food protein of optimal nutritional value according to biological criteria. In the entire gamut of foods the only one specifically designed (one might say divined) as food for man, namely human milk, would appear to be a logical choice. This has indeed been recommended as the standard by Kuhnau (1949), Nehring and Schwerdtfeger (1951) and others. However the author has preferred to follow the example of Mitchell and Block (1946) and adopt whole egg protein as the reference standard. It is as nearly completely utilized by the rat, the dog and man as any food protein and is not significantly enhanced in biological value by supplementation with any of the amino acids. The adoption of an "ideal" protein as a standard involves the



corollary assumption that the percentage of an essential amino acid in a protein in excess of its percentage in the standard can be ignored. In other words the value of the food protein in this respect is regarded as equal to, but not greater than the standard.

#### IV DERIVATION OF AN INTEGRATED ESSENTIAL AMINO ACID INDEX

Thus the two more or less arbitrary assumptions upon which the calculation of an integrated essential amino acid index is based are that the minimum ratio of essential amino acid content relative to that of standard protein is 1%, and the maximum 100%.

The Essential Amino Acid Index (EAA index) may be defined as the geometric mean of "the egg ratios" i.e. the ratios of the essential amino acids in a protein relative to their respective amounts in whole egg protein

$$\text{EAA Index} = \sqrt[n]{\frac{\text{Lys}_p}{\text{Lys}_s} \times \frac{\text{Try}_p}{\text{Try}_s} \times \frac{\text{His}_p}{\text{His}_s}}$$

in which the subscript  $p$  refers to the food protein,  $s$ , the standard protein (whole egg) and  $n$ , the number of amino acids (counting pairs such as methionine and cystine as one) entering into the calculation. It is generally of interest to inspect the individual egg ratios and hence these are recorded in the computation of EAA indexes.

Amino acid contents are usually expressed on a nitrogen = 16 basis. However EAA indexes may be computed from the amino acid content relative to the total nitrogen in both the food protein and the standard. This avoids the necessity of applying the conventional factors 6.25, 6.38 or 5.7 for converting nitrogen to protein. The simplicity of the computation as conducted logarithmically is illustrated by the model table used in the author's laboratory shown in Table I. The figures entered in roman type are printed on the form whereas those shown in italics are entered by hand. Occasionally certain amino acid values are not available but reasonably close approximations to the EAA indexes may be obtained even omitting these values provided of course, that the appropriate value for  $n$  is used. When data for cystine and tyrosine are lacking as is not infrequently the case, methionine and phenylalanine, respectively may be considered individually.

In the original paper on the EAA index (Oser 1951) the author used the analytical values reported by Block and Bolling (1951) for the essential amino acid content of whole egg protein. This practice was subsequently followed by Mitchell (1954a). However the values summarized recently by Orr and Watt (1957) which were derived from improved

TABLE I  
CALCULATION OF ESSENTIAL AMINO ACID INDEX<sup>a</sup>

Amino acid (AA)	Whole egg				Food (cow milk)			
	(a) % (N = 16)	(b)		(c) 1	(d)		(e) Egg ratio 100 (c d)	(f) Log (e)
		gm AA	gm N		gm AA	gm N		
Lysine	6.40	0.100	2.50	0.96	0.096	121.0	(2.0000)	
Tryptophan	1.65	0.103	9.71	0.090	0.090	87.1	1.9415	
Isoleucine	6.60	0.415	2.41	0.407	0.407	99.1	1.9917	
Valine	7.42	0.464	2.16	0.438	0.438	91.6	1.9759	
Arginine	6.56	0.410	2.44	0.233	0.233	56.9	1.7551	
Methionine	3.14	0.196		0.156	0.156			
Cystine	2.34	0.146		0.057	0.057			
Methionine + Cystine	5.48	0.342	2.92	0.213	0.213	62.2	1.7938	
Threonine	4.98	0.311	3.22	0.294	0.294	94.7	1.9764	
Leucine	8.90	0.550	1.82	0.626	0.626	113.9	(2.0000)	
Phenylalanine	5.78	0.361		0.309	0.309			
Tyrosine	4.30	0.269		0.325	0.325			
Phenylalanine + Tyrosine	10.08	0.630	1.59	0.634	0.634	100.8	(2.0000)	
Histidine	2.40	0.150	6.67	0.168	0.168	112.1	(2.0000)	
Sum of log (Egg Ratios) = $\sum (f)$ = 19.4344 Mean log Egg Ratios = $\frac{\sum (f)}{10}$ = 1.9434 EAA Index = Anthlog $\sum [(f)/10]$ = 87.8								

<sup>a</sup> Food cow milk 0.55% N  $\times$  6.38 = 3.5% protein

and more numerous microbiological assays have been used as the basis for computing the indexes tabulated in the Appendix to this presentation. In Table II are shown the assumed values for the essential amino acid content of whole egg protein according to both sets of published data. It is of incidental interest to note that the Orr and Watt data for whole egg are in closer agreement than Block and Bollings with the

TABLE II  
ESSENTIAL AMINO ACID CONTENT OF WHOLE EGG PROTEIN

Amino acid	Data of Block and Bolling (1951)	Data of Orr and Watt (1957)		
	Per cent (N = 16)	Number of samples assayed	gm/gm N	Per cent (N = 16)
Lysine	7.0	26	0.400	6.40
Tryptophan	1.5	27	0.103	1.65
Isoleucine	7.7	16	0.415	6.60
Valine	7.2	16	0.464	7.42
Arginine	6.6	21	0.410	6.56
Methionine	4.0	34	0.196	3.14
Cystine	2.4	24	0.146	2.34
Methionine + Cystine	6.4		0.342	5.48
Threonine	4.3	27	0.311	4.98
Leucine	9.2	16	0.550	8.80
Phenylalanine	6.3	26	0.361	5.78
Tyrosine	—	18	0.269	4.30
Phenylalanine + Tyrosine	—		0.630	10.08
Histidine	2.4	26	0.150	2.40

best average values selected in the Rutgers report (Allison *et al.*, 1950). The differences in any case are small and fall well within the reported range of variation for the amino acid content of whole egg. Table III is intended to demonstrate the small magnitude of the differences in EAA index as affected by the selection of amino acids regarded as essential or by the source of assay data for the amino acid content of either the standard or the food proteins. It is evident from this table that either Block and Bollings or Orr and Watt's data for the reference protein may be used in computing EAA indexes. For all practical purposes no differences were found in this series of typical food proteins [cf. columns (d) and (e)].

It is suggested that pending possible future agreement on standard values by the Food and Agriculture Organization of the United Nations or a similar official agency the data summarized by Orr and Watt

TABLE III  
ESSENTIAL AMINO ACID INDICES OF FOOD PROTEINS COMPUTED IN VARIOUS WAYS

(a)	(b)	(c)	(d)	(e)	(f)	(g)
	As in Col (a) plus His Arg <sup>c</sup>	As in Col (a) plus His Tyr <sup>d</sup>	As in Col (a) plus His Arg Tyr	As in Col (a) plus His Arg <sup>c</sup>	As in Col (a) plus His Arg Tyr	As in Col (a) plus His Arg Tyr
Eight essential amino acids <sup>a</sup>						
Standard protein whole egg						
Standard protein human milk						
Food assayed	Assay data for standard		Block and Bolling		Assay data for standard	
	Assay data for food		Block and Bolling		Assay data for food	
Beef muscle	85	88	87	84	84	91
Corn whole	67	70	71	65	67	76
Gelatin	22	27	26	26	25	27
Milk cow	93	90	90	86	88	96
Milk human	97	94	94	85	87	(100)
Oats rolled	73	77	78	74	72	78
Soybean	82	85	85	83	83	89
Wheat, flour	57	60	60	61	61	69
Wheat whole	65	67	67	63	64	72
Yeast, primary	82	84	81	79	82	91

<sup>a</sup> The eight essential amino acids included in all computations are lysine tryptophan isoleucine valine methionine plus cystine threonine leucine and phenylalanine

<sup>b</sup> Rose (1938)

<sup>c</sup> Nehring and Schwerdtfeger (1951)

<sup>d</sup> Mitchell (1954a)

and more numerous microbiological assays, have been used as the basis for computing the indexes tabulated in the Appendix to this presentation. In Table II are shown the assumed values for the essential amino acid content of whole egg protein according to both sets of published data. It is of incidental interest to note that the Orr and Watt data for whole egg are in closer agreement than Block and Bollings with the

TABLE II  
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Phenylalanine + Tyrosine	—		0.630	10.08
Histidine	2.4	26	0.150	2.40

best average values selected in the Rutgers report (Allison *et al.* 1950). The differences in any case are small and fall well within the reported range of variation for the amino acid content of whole egg. Table III is intended to demonstrate the small magnitude of the differences in EAA index as affected by the selection of amino acids regarded as essential or by the source of assay data for the amino acid content of either the standard or the food proteins. It is evident from this table that either Block and Bollings or Orr and Watt's data for the reference protein may be used in computing EAA indexes. For all practical purposes no differences were found in this series of typical food proteins [cf. columns (d) and (e)].

It is suggested that pending possible future agreement on standard values by the Food and Agriculture Organization of the United Nations or a similar official agency, the data summarized by Orr and Watt



(Table II) be used since these are derived from the most up to date and extensive series of assays

Mitchell has modified (1954a) the author's original proposal for calculating EAA indexes (Oser 1951) by disregarding arginine,  $\text{NH}_2$  being nonessential, and including tyrosine together with phenylalanine (in analogy with the pairing of cystine with methionine) because of its partial replacement value. The negligible overall effect of these modifications may be seen by comparing columns (b) and (c) in Table III. Even restricting the essential amino acids to the eight shown by Rose *et al.* (1954) to be required for nitrogen balance in young adult males (but including cystine with methionine) has little influence on the indexes [column (a)].

The author is inclined now to accept Mitchell's recommendation to include tyrosine with phenylalanine but not to consider arginine as dispensable until further data on human beings in various physiological states indicates this to be justified [cf columns (d) and (f)]. In any event the differences in indexes are not greatly influenced by whether or not arginine is included.

It is of interest to compare the values for EAA indexes calculated with reference to egg protein to those based on human milk protein as the standard. This comparison is shown in columns (f) and (g) of Table III. It will be noted that somewhat higher indexes are consistently obtained in relation to human milk protein, the increases in the foods listed ranging from 8 to 15%.

A high degree of correlation between EAA indexes and reported biological values are obtained regardless of whether human milk or egg protein is used as the standard. Mitchell has reported the following regression equation based on 48 such comparisons with a correlation coefficient ( $r$ ) of + 0.948

$$\text{Biological value} = 1.0747(\text{EAA index}) - 13.74 \quad (1)$$

The author's smaller series (Oser 1951) comprised 29 comparisons and yielded the following equation

$$\text{Biological value} = 1.1403(\text{EAA}) - 8.415 \quad (2)$$

On the basis of the foregoing discussion indicating that the methods of computation employed in both of these series of data yield essentially the same results, the following equation weighting the two series of 77 comparisons is derived

$$\text{Biological value} = 1.09(\text{EAA}) - 11.73 \quad (3)$$

As Mitchell has pointed out the correlation between the EAA index

and the biological value is so consistently high that estimates of the latter may be made from essential amino acid assay with a degree of reliability greater in the long run than is obtainable in the usual biological assay employing 10 rats per group. However it is necessary to recall that this method of estimation is predicated upon the availability to the animal organism of the microbiological or chromatographically determined amino acids. Factors such as insolubility or heat treatment which impair the degree or rate of digestibility and absorption of protein militate against the use of this procedure in all cases. Proteins like hemoglobin and keratin are poorly utilized and the utilization of certain food proteins like soybean or casein are affected by processing conditions. It is not valid to assume that the entire amino acid content of these proteins as estimated by nonbiological methods is physiologically available.

The table shown in the Appendix is based on the analytical data of Orr and Watt and the application of Eq. (3) above the resultant values being rounded off to the nearest whole numbers. Whereas biological values for many of these food proteins are to be found scattered in the literature those cited in the last column were taken from Mitchell's paper (1954a) merely to illustrate the general agreement between calculated and observed estimates even though the samples upon which these estimates are based differed.

## V LIMITATIONS OF THE ESSENTIAL AMINO ACID INDEX

As has been previously pointed out factors which affect the degree or rate of digestibility of proteins influence not only their over all nutritive value but specifically their biological value in the Mitchell sense. Hence any index of utilization based on the total content of essential amino acids in a protein may tend to overestimate the true value.

Sheffner and associates (1956) have attempted to overcome this difficulty by basing the computation of the index on the pattern of essential amino acids in enzymatic digests of proteins rather than on the total essential amino content in complete hydrolyzates. For this purpose they used peptic followed by tryptic and ereptic digestion for periods of 24, 24 and 72 hours respectively. The essential amino acid content of these digests is computed according to the authors method as modified by Mitchell [cf. column (b) Table III] and the result is expressed as a PDR index (Protein Digestion Residue index). The data for eight series of comparisons show that the PDR index corrected for digestibility, correlates quite closely with the biological value. However it is questionable whether the improvement in this relationship over that of the EAA index itself justifies the considerably greater volume of work involved. Only in those cases where there would appear to be reason for believing that



digestibility or utilization were significantly impaired would this technique be warranted. In only two instances (Labco casein and white flour) cited by these authors did this appear to be the case (Compare also the values for casein shown in the Appendix).

It is of interest to point out that Kuiken and Lyman (1948) observed in metabolic studies that all of the essential amino acids were completely available in roast beef, wheat and peanut flour whereas the amino acids in cottonseed flour were less available, values ranging from 64% in the case of lysine to 94% in the case of arginine. If the entire essential amino acid content of cottonseed flour were available the EAA index would be approximately 71 whereas that of the available fraction only is 58.

## VI SPECIAL APPLICATIONS OF THE ESSENTIAL AMINO ACID INDEX

A useful property of the EAA index as a tool for predicting biological values is that it permits estimates to be made for combinations of proteins or for proteins supplemented with individual amino acids or combinations thereof. For instance it has been computed that the addition of 0.55% lysine to white flour would affect an increase in the index from 60 to 69, corresponding to an increase in the expected biological value from 54 to 64 or approximately the value for whole wheat. In making such calculations it is of course necessary to express the essential amino acid content of the final mix or supplemented proteins on an isonitrogenous basis.

Another example of the use of this procedure is the estimation of the EAA index for mixtures of proteinaceous foods such as that employed by Scrimshaw for the prevention of protein malnutrition in Guatemala (Behar *et al.* 1957). This consists of corn, sesame meal, cottonseed pressed cake and torula yeast mixed in the proportions of 50:35:9:3. The EAA index of these components are 72, 75, 72 and 80 respectively whereas that of the mixture is 79.

The EAA index for corn flakes according to recent analyses is 60 (predicted  $\Pi V = 54$ ). Ordinarily one ounce of this breakfast cereal is taken with 8 fluid ounces of milk which has an EAA index of 88. The proteins from these sources are in the approximate ratio of 1:3.5 and the EAA index for the combination is calculated to be 84 with a predicted biological value of 80.

In computing the essential amino acid indexes of combinations of proteins their full individual contributions are taken into account before disregarding that proportion of the egg ratio of the combination in excess of 100%.

## VII SUMMARY

A system of rating dietary proteins has been described based in part on the rationale that the efficiency of utilization is a function of the product of the essential amino acids made available at the site of synthesis. Designated the Essential Amino Acid Index (EAA index) it is the geometric mean of the ratios of the essential amino acids in the food protein relative to their content in a highly nutritive reference protein viz whole egg. The basis for the choice of this "standard" and of the pertinent analytical data is discussed. Application of the regression equation

$$\text{Biological value} = 1.09 (\text{EAA}) - 11.7$$

yields close agreement between predicted and observed biological values.

The EAA index not only integrates all the essential amino acids into the calculation but permits estimation of the effect of amino acid or protein supplementation on biological values.

Certain limitations in the use of the index are discussed particularly those cases where the utilization of amino acids is influenced by factors which affect the rate of enzymatic release of essential amino acids.

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## APPENDIX

## ESSENTIAL AMINO ACID INDEXES AND BIOLOGICAL VALUES OF FOOD PROTEIN

Item number and description <sup>a</sup>	EAA	Biological v:
	Index <sup>b</sup>	Predicted <sup>c</sup> Ob
Milk		
1 Cow whole nonfat evaporated or dry	88	84
3 Human	87	83
Milk products		
5 Buttermilk	88	84
6 Casein	88	84
7 Cheese cheddar other ripened cheeses <sup>d</sup> and processed cheese foods	86	82
8 Cottage	86	82
9 Cream cheese	82	77
10 Lactalbumin	89	85
11 Whey dried	69	61
Eggs chicken		
12 Whole raw or dried	(100)	97
13 Whites raw or dried	95	91
14 Yolks raw or dried	93	89
Meat		
15 Beef cuts <sup>e</sup> fresh or canned	84	80
16 Lamb cuts <sup>e</sup> fresh or canned	84	78
17 Pork cuts fresh pork <sup>e</sup> raw or canned	83	79
20 Ham and other cured pork raw cooked or canned	81	77
22 Veal cuts fresh or canned	83	79
Poultry		
23 Chicken muscle without skin	82	78
24 Duck muscle without skin	82	78
Fish and shellfish		
26 Fish raw or canned	80	76
27 Shellfish shrimp including prawns raw or canned	67	61
28 Other shellfish	76	71
29 Brains	85	81
31 Fish flour	77	73
32 Gelatin	25	16
33 Gizzard chicken	75	70
34 Heart	86	82
35 Kidney	86	82
36 Liver	89	85
41 Sausage containing liver	83	78
42 Other sausage	77	72
43 Tongue fresh or smoked	82	77

## APPENDIX (Continued)

Item number and description*	EAA Index <sup>b</sup>	Biological value	
		Predicted <sup>c</sup>	Observed
Legume seeds and their products			
Beans includes kidney navy pinto red others			
45 Raw	80	75	
46 Baked with pork canned	73	68	
47 Baked with tomato sauce canned	65	60	
48 Black gram ( <i>Phaseolus mungo</i> )	80	70	
49 Broad beans ( <i>Vicia faba</i> )	70	65	
50 Chickpeas ( <i>Cicer arietinum</i> )	77	72	
51 Cowpeas ( <i>Vigna</i> spp.)	79	74	
53 Lentils ( <i>Lens culinaris</i> )	71	65	
54 Lima beans ( <i>Phaseolus lunatus</i> )	78	74	
57 Mung beans ( <i>Phaseolus aureus</i> )	70	65	
58 Peanuts flour meal peanut butter ( <i>Arachis hypogaea</i> )	69	64	57
59 Peas ( <i>Pisum sativum</i> )	81	77	
61 Soybeans and flour ( <i>Glycine max</i> )	83	78	75
63 Soybean milk	86	82	
Common nuts			
65 Almonds ( <i>Prunus amygdalus</i> )	64	58	
66 Brazil nuts ( <i>Bertholletia excelsa</i> )	69	64	
67 Cashews ( <i>Anacardium occidentale</i> )	64	58	
68 Coconut and other palm family nuts and meals <sup>d</sup>	68	63	
69 Filberts ( <i>Corylus</i> spp.)	68	62	
70 Pecans ( <i>Carya illinoensis</i> )	76	71	
71 Walnuts English or Persian ( <i>Juglans regia</i> )	70	65	
Seeds and seed meals			
78 Cottonseed flour and meal ( <i>Gossypium</i> spp.)	72	67	64
83 Sesame seed and seed meal ( <i>Sesamum indicum</i> )	73	68	71
84 Sunflower seed meal ( <i>Helianthus annuus</i> )	71	66	65
Grains and their products			
85 Barley ( <i>Hordeum vulgare</i> )	66	60	
86 Bread white made with refined wheat flour and 4% nonfat dry milk flour basis	64	58	
88 Buckwheat flour ( <i>Fagopyrum esculentum</i> )	73	68	
93 Corn cornmeal grits ( <i>Zea mays</i> )	67	61	62
Corn products			
94 Flakes	60	54	
95 Germ	73	67	78

## APPENDIX (Continued)

Item number and description <sup>a</sup>	EAA Index <sup>b</sup>	Biological value	
		Predicted <sup>c</sup>	Observed
96 Gluten	63	57	
97 Hominy	68	62	
100 Tortilla	66	60	
101 Zein	31	22	
105 Pearl millet ( <i>Pennisetum glaucum</i> )	75	70	
107 Oats oatmeal rolled oats ( <i>Avena sativa</i> )	72	67	65
109 Rice ( <i>Oryza sativa</i> ) brown converted white	73	68	70
112 Rye ( <i>Secale cereale</i> ) whole grain and flours of different extractions	68	62	
113 Sorghum ( <i>Sorghum vulgare</i> )	70	65	
115 Wheat ( <i>Triticum aestivum</i> ) whole grain and whole grain flour	64	58	67
117 White flour	61	54	52
Wheat products			
118 Bran	71	66	
122 Germ	74	69	75
123 Gluten	60	54	
124 Macaroni or spaghetti	55	48	
125 Noodles (contain egg solids)	67	61	
126 Shredded wheat	65	59	
Vegetables immature seeds			
146 Corn ( <i>Zea mays</i> )	72	66	
147 Cowpeas ( <i>Vigna</i> spp.)	79	74	
148 Lima beans large and small seeded varieties ( <i>Phaseolus lunatus</i> including var <i>macrocarpus</i> )	84	79	
149 Peas raw or canned ( <i>Pisum sativum</i> )	64	58	
Leafy vegetables			
152 Brussels sprouts ( <i>Brassica oleracea</i> var <i>gemmifera</i> )	64	58	
153 Cabbage ( <i>Brassica oleracea</i> var <i>capitata</i> )	56	49	
157 Kale ( <i>Brassica oleracea</i> var <i>acephala</i> )	61	54	
161 Spinach ( <i>Spinacia oleracea</i> )	82	77	
162 Turnip greens ( <i>Brassica rapa</i> )	76	71	
Starchy roots and tubers			
165 Cassava root and flour ( <i>Manihot esculenta</i> )	54	47	
166 Potatoes ( <i>Solanum tuberosum</i> )	65	59	
167 Sweet potatoes ( <i>Ipomoea batatas</i> )	82	78	
168 Taro ( <i>Colocasia</i> spp.)	81	76	
Other vegetables			
171 Asparagus ( <i>Asparagus officinalis</i> )	62	56	
172 Beans snap ( <i>Phaseolus vulgaris</i> )	66	60	

## APPENDIX (Continued)

Item number and description <sup>a</sup>	EAA Index <sup>b</sup>	Biological value	
		Predicted <sup>c</sup>	Observed
173 Beets ( <i>Beta vulgaris</i> )	39	31	
174 Broccoli ( <i>Brassica oleracea</i> var <i>botrytis</i> )	68	60	
175 Carrots ( <i>Daucus carota</i> )	59	52	
176 Cauliflower ( <i>Brassica oleracea</i> var <i>botrytis</i> )	73	68	
182 Eggplant ( <i>Solanum melongena</i> )	57	50	
186 Okra ( <i>Hibiscus esculentus</i> )	65	59	
190 Pumpkin ( <i>Cucurbita pepo</i> )	54	47	
195 Tomatoes and cherry tomatoes ( <i>Lycopersicon esculentum</i> and <i>L. esculentum</i> <i>ceraiforme</i> )	48	41	
Miscellaneous food items			
199 Yeast Bakers	80	76	
200 Yeast Brewers dried	83	79	
201 Yeast Primary dried ( <i>Saccharomyces cerevisiae</i> )	82	77	
202 Torula yeast ( <i>Torulopsis utilis</i> )	88	85	

<sup>a</sup> As listed by Orr and Watt (1957)

<sup>b</sup> Computed from data of Orr and Watt (1957) of Table III column (f)  
 $BV = 1.09 (EAA) - 11.73$  See page 298

<sup>c</sup> Includes such kinds as Blue Limburger and Swiss

<sup>d</sup> Based on data from many cuts

<sup>e</sup> Including coconut (*Cocos nucifera*) babassu (*Orbignya speciosa*) palm cohune (*Orbignya cohune*) and palm nut (*Elaeis guineensis*)

graphical or economic background. It is generally agreed that nutrition and dietary habits or customs play an important role in these correlations. Limited intake of good quality proteins has long been held responsible for the occurrence of substandard anthropometric patterns. This is particularly true if we consider the view held by some that low protein intake in countries like China and India (in contrast to Australia and the United States, where protein consumption is high) is responsible for existing differences in stature, longevity, and general health (Albanese, 1956a). For example, life expectancy in Australia and the United States was about twice that of the populations of China and India (Table I). Recent improvements in sanitation and medical care

TABLE I  
RELATION OF NATIONAL DIETS TO STATURE AND LONGEVITY<sup>a</sup>

	Australia	United States	China
Total calories per day	3128	3249	2201
Livestock % calories	40-45	35-40	1-5
Cereals and potatoes % calories	30-40	30-40	70-80
Total protein intake gm per day	90	88	68
Animal protein per cent	85	57	7
Average height cm	172	170	158
Average weight kg	77.2	70.0	54.3
Life expectancy yr	65	64	30
Total calories per gram of protein intake	35	37	32

<sup>a</sup> From Albanese (1956a).

shown that these are important factors in longevity (UN Secretariat, 1958). However, it is well known and amply supported by statistical evidence that racial differences with regard to height and weight can be readily overcome with an improved nutritional environment. Many evidences of this phenomenon in native populations of Africa and Asia have been presented also by Flodin (1953).

The occurrence of nutritionally related changes in anthropometry is indicated by examination of some of our own vital statistics. It is generally conceded that compared with those of other countries, the diets in the United States are generous and that the range in variety of food products is unusually large. Improvements in stature and body weight among immigrants to the United States have been attributed statistically to the better diets obtainable here. To wit: the second generation Japanese in California and of Central Europeans in the large American cities are of larger stature and better physique than their parents. At the same time American women entering colleges are found to average

more than an inch taller than those in the same colleges thirty years ago. The average stature of Harvard men has increased about two inches in the last sixty years.

### A. STANDARDS

Figures on the relationship between body weight and other body measurements of North American adults go back scarcely a century. According to Marks (1956) the first table prepared in 1897 was based upon 74 162 male applicants accepted for life insurance in the United States and Canada. The first extensive tables for women appeared in 1908 and were based upon measurements of about 60 000 cases insured in the previous decade. Such data collected at irregular intervals since those years indicate that average weights specific for height and age were fairly stable over long periods except as noted above at the younger ages. Mortality and morbidity statistics suggest that the standards of weight long in vogue in this country and still widely used are too generous. A new study of life insurance experience now under way, will provide the basis of new standards.

Growth patterns are frequently employed in the evaluation of the quantitative and qualitative nutrient needs of children. To expedite and quantitate these evaluations it is helpful for the investigator to have a graph or table showing the norms of growth for infants and children. Several compilations are now in use in this country. Wetzel (1946) through his widely used grid based on heterogeneous data has made a distinct contribution to the clinical interpretation of physical measurement of height and weight. Meredith (1949-1955) has devised growth charts based on the distribution of height and weight measurements made between 1950 and 1940 of Iowa City children. Rueda Williamson (1958) has recently reviewed the available methods for assessment of growth and development of children with special reference to a modified form of the Wetzel Grid. Naimark (1957) has collected the available data on American children into a convenient Growth Spectrum<sup>1</sup>. This chart visually emphasizes by colored weight and height zones differences in growth patterns.

#### 1. Applications

Anthropometric standards of reference are particularly useful in the objective interpretation of nutritional survey data from economically underdeveloped areas. Thus the problem of ascertaining the preferable adjunct to a predominantly cereal diet can often be determined in the human where its solution is of the greatest importance (Widdowson and

<sup>1</sup> Copies of this chart may be obtained from the White Laboratories Inc. Kenilworth New Jersey.



often results in such severe dehydration that restorative fluid therapy must be applied. Massive infections also reduce the normal water content of tissues. Cardiovascular diseases in advanced stages cause edema

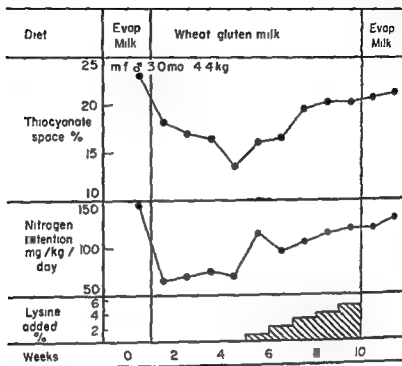


FIG 1 Effect of a wheat gluten diet and lysine supplementation on nitrogen retention and thiocyanate space

Malnutrition arising from a suboptimal overall intake of food or starvation at first induces a loss of body water, and if prolonged an accumulation of water in the tissues—generally referred to as nutritional edema. In some phases of convalescent malnutrition this edema may be subclinical in nature. Higgons and Albanese (1957) reported that measurements in 44 adult convalescents, receiving a high protein supplement for 21 days without overt signs of edema showed an average decrease in thiocyanate space of 1686 ml (equivalent to 37 lb). During the same period of time the scales indicated an average increase in gross body weight of only 20 lb, whereas in fact these patients had experienced an average increase of at least 57 lb in physiologic body mass.

### 3 Age

Advancing years have been found to cause a progressive loss of body water which is associated with an almost proportionate increase in fat deposition. Although no accurate figures on the magnitude of this dehydration in man are available, some approximations have been made

from water balance measurements (Brozek, 1952). The proclivity of increased fat deposition with age is well known and it seems to take place at the expense of losses in body water (Fig. 2).

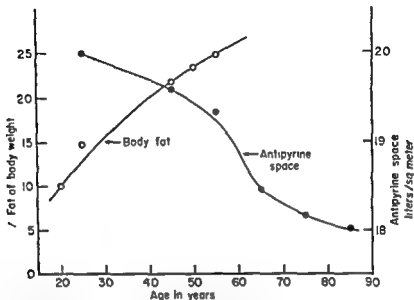


FIG. 2. Relationship of body fat and antipyrine space to age of man. Adapted from the data of Brozek (1952) and Watkin (1958).

### C. SUMMARY

The knowledge and consideration of the factors which influence body composition have long troubled this author in the interpretation of nutritional data derived from his own studies and those of other investigators. This concern has led to many as yet unpublished efforts on the measurement of body tissue compartments during the test pre- and postcontrol periods of many dietary studies. Since most of these studies have been performed on human subjects, only nondestructive procedures could be employed. This limitation permitted the consideration of only the specific gravity or dilution techniques. The specific gravity methods so well investigated and reported by Behnke (1953) cannot be applied readily to young children, the aged, or convalescent adults. Of the reported dilution methods, we have had extensive experience with the thiocyanate, Evans blue, and antipyrine variants, but not heavy water. In our investigations, serial measurements with these agents, which are necessary in metabolic studies, have proved too bothersome for our category of subjects, who are typical of any civilian hospital population. Furthermore, the interpretation of the data obtained by these methods in terms of inter- or intracellular body water changes

cannot be made readily in biochemically or physiologically abnormal subjects. It is obvious that newer techniques must be explored to facilitate the determination of changes in body composition which are clearly so important a factor in the measurement of gross body weight. The lack of such modalities constitutes a serious obstacle in the further advance of nutritional knowledge.

## II BLOOD PROTEINS

The laboratory methods most widely used in appraising nutritional status are the determination of the plasma proteins and the hemoglobin content of the blood. The use of these criteria as a measure of protein intake and metabolism derived early support from the studies of Holman and his associates (1934), who postulated that blood proteins take part in the dynamic equilibrium existing between all the body proteins. Their findings have agreed in the main with the observations of Weech (1938, 1942) and of Melnick *et al* (1936). The direct incorporation of ingested amino acids into plasma proteins has also been shown by Schoenheimer and his co-workers (1942) by the feeding of isotopic amino acids. By this technique these workers demonstrated that the various plasma protein fractions participate in general metabolic reactions to about an equal extent. Hemoglobin anabolism is known to suffer under certain conditions when the protein intake is inadequate (Youmans, 1945). The Ortens (1943) found that chronic anemia could be produced in rats by feeding them a diet low in protein. Albanese and co-workers also demonstrated that dietary deficiencies of tryptophan (1943) and methionine (1946a) produced marked anemia and hypoproteinemia in young and mature rats. Subsequent studies by Shemin (1948) have demonstrated the participation of glycine and other two or three carbon compounds in the formation of hemoglobin. It is clear from this and other available evidences that the blood proteins participate dynamically in the metabolic pool and should therefore reflect by their amount a measure of the nutritional state of the organism.

Unfortunately, in an attempt at technical simplification widespread use has been made of concentration measurements as an index of total circulating plasma proteins or hemoglobin. This usage is based on the false assumption that the circulating fluids of the body are constant in volume. Since as noted in the previous section the volume of circulating plasma is subject to considerable variation in relation to growth, age, health and acute and chronic disease it is evident that significant changes in total quantities of circulating blood proteins may be masked if protein concentration is measured without regard for expanding or contracting blood volume. Peters (1942) has aptly pointed out that

three dimensional functions cannot be evaluated by two dimensional measurements and that hence alterations in total circulating protein and plasma volume cannot be estimated from percentage protein concentrations. It is obvious from these considerations that the circumstances under which plasma protein and hemoglobin levels may serve as criteria of protein intake or nutritional status will have to be re-evaluated in the light of newer knowledge.

## A BIOSYNTHESIS AND DIETARY PROTEIN QUALITY

### 1 *Experimental Animals*

The efficiency with which dietary proteins are used for the formation of blood proteins has been employed extensively by Menden and Whipple (1940) as a measure of their nutritive value. The technique as developed by this group at the University of Rochester, is basically simple. Dogs are depleted of their blood proteins as rapidly as is consistent with their well being. A zero protein diet plus frequent blood removal achieves depletion of both hemoglobin and plasma proteins within a 3 to 4 week period. The dogs are then placed on a test protein diet weighed daily and their protein intake accurately calculated. Plasma protein and hemoglobin levels and total blood volume are determined and corrected for the amounts of blood protein actually removed by bleeding. From these figures the amounts of plasma proteins and hemoglobin produced weekly can be calculated and compared with the dietary intake. A number of test proteins were fed. These included beef heart and muscle casein egg yolk egg white whole egg lactalbumin pork liver peanut flour canned pink Alaska salmon and wheat gluten. The results of these studies are given in Table III. These figures are averages of the data obtained from 2 to 5 dogs fed the several proteins and they are listed in the descending order of total blood protein formation in terms of output per week. It will be observed that considerable discrepancies exist between blood protein production values and gain or loss of body weight. This is particularly true of wheat gluten which ranks fourth in the production of blood proteins and yet produced the greatest weight loss. This weight loss may be associated with excessive loss of body fluids (Fig. 1).

In a series of studies sponsored by the Bureau of Biological Research of Rutgers University in which five reference proteins (egg white lactalbumin whole egg casein and wheat gluten) were evaluated by different methods of assay similar paradoxical findings were obtained in regard to the nutritional value of these proteins and especially with wheat gluten (Allison 1949). Chow (1950), working with protein depleted dogs found a marked lack of correlation between nitrogen





proteins was slowly restored to the norm from the aberrant values of 70-92% arginine. No significant alteration in the albumin globulin ratios was observed throughout the course of these studies. From this it would appear that the compositional change involved both major plasma fractions. The nitrogen retention of all infants was restored to normal values when the wheat gluten diet was supplemented so that the lysine intake ranged from 185-220 mg per kilogram daily. Since the percentage plasma protein levels were not altered by this dietary sup

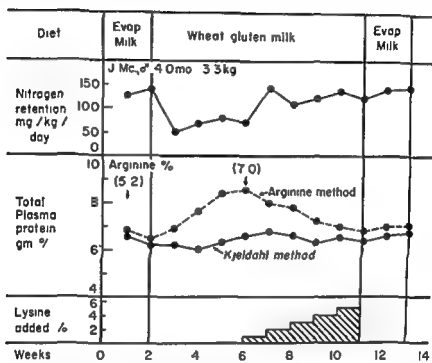


FIG. 3 Effects of a wheat gluten diet on the nitrogen retention, plasma protein levels, and arginine content of the plasma proteins. The values in parentheses denote calculated arginine content of the plasma proteins.

plement it must be concluded that measurements of these blood components do not constitute an adequate criterion of lysine intake in the infant.

The dietary lack of tryptophan (Albanese *et al.* 1947b), methionine (Albanese *et al.* 1949a) or isoleucine (Albanese *et al.* 1948) has been found to cause a drop in nitrogen retention as well as a decrease of 20% or more in plasma protein levels of infants within 10 to 16 days. The finding that plasma proteins are formed at an apparent normal rate in the face of an inadequate lysine intake (Fig. 3) suggests that synthesis could be maintained by one of two processes: (a) substitution of arginine in these proteins or (b) an increase in a plasma protein fraction or fractions rich in arginine. On the basis of available collateral evidence





decidedly below normal levels. In studies on women in Rochester, York, he found further that 204 clinic patients showed consistently higher plasma protein levels throughout the pregnancy period than 210 private patients. This occurred in spite of the fact that the protein intake of the private patients was approximately 10% higher than that of the clinic patients.

Recent data of Albanese and co workers (1958) on adults shows that there exists relatively poor correlation between total plasma protein

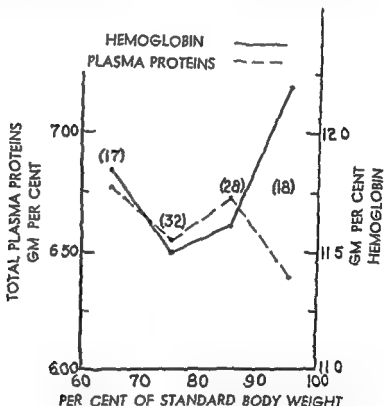


FIG. 4. Blood protein levels and body weight variations of adults 40-73 years of age.

levels and deviation from desirable body weight (% S), but good correlation between hemoglobin concentration and body weight in the 100-70% S range. The experiments of Madden and Whipple (1952) suggest that this observed divergence of hemoglobin content and body weight in the 70-60% S range (Fig. 4) may constitute an expression of the hemoconcentration effects of severe malnutrition.

Depletion of plasma protein specifically albumin occurs frequently in the aged (Rafsky *et al.*, 1952). Chesrow and co workers (1958) have shown that the total serum proteins of 41 adults (70-94 years) were significantly lower than that of 13 younger adults (20-35 years). The

was caused primarily by a decrease in the albumin level of the aged group. The explanation for this difference is not clear. Chinn and his associates (1956) were unable to demonstrate any discrepancy between the rate of digestion and absorption of an  $^{131}\text{I}$  labeled albumin test meal in aged and young people. However both Kountz *et al* (1951) and Albanese *et al* (1957) have demonstrated that negative nitrogen balances may occur subclinically in the elderly.

In the light of the foregoing the question arises as to what nutritional significance can be given to plasma protein and hemoglobin concentration values. The clinical value of plasma protein concentration levels and albumin globulin ratios in some diseases has been well established (Poh 1951). In the authors experience these measurements are an invaluable aid in following the nutritional effects of nephrosis in children (Albanese *et al* 1949b). Similarly the hemoglobin determination has proven itself an indispensable tool in the study and treatment of anemias (Wintrobe 1949). In most of these instances where the clinical and nutritional state of the patient correlates well with the blood protein picture the problem of interpretation and therapy is clear. The paradoxical findings seem to occur predominantly in malnourished individuals but they are not associated with any specific disease. Available evidence suggests that it is this subclinically ill group who might benefit most readily from dietotherapeutic measures (Cannon 1944). Unfortunately we have no ready means at present for detecting the need for such measures. Nitrogen balance or fluid space determinations which in conjunction with blood protein concentration measurements might aid in the detection of dietary defects are not always practicable.

### C SUMMARY

It is abundantly clear from the evidence that concentration levels of hemoglobin and serum or plasma proteins are not always dependable criteria of the nutritional state in some individuals. Examination of the available evidence indicates that this lack of correlation may arise from (a) hemoconcentration or hemodilution effects (b) compensatory compositional changes of the blood proteins and (c) an asymmetric rate of synthesis of various tissue proteins. We feel that the existence of these phenomena should be more generally recognized and that basic studies should be undertaken to attempt a resolution of the anomalies.

### III NITROGEN BALANCE

The simplest and oldest chemical method used to evaluate the nutritional properties of proteins in an animal is to determine the difference between nitrogen intake and nitrogen excreted. This difference, called nitrogen balance, shows whether an animal is gaining or losing bodily nitrogen. If the nitrogen intake equals the total nitrogen output, the balance hovers about zero and the animal is said to be in *nitrogen equilibrium*. This condition is attained in the normal well fed adult. In the well fed immature organism which is endowed with a growth potential involving the formation of tissues the nitrogen balance is always positive, that is, the nitrogen intake exceeds the nitrogen output. This indicates that nitrogen is being retained for synthesis of body tissues. In man, a maximum positive value is achieved during the first six months of life, and decreases steadily in a logarithmic manner with age.

#### A THEORY

A very useful mathematical exposition (Fig 5) of the theory of the nitrogen balance method was set forth some thirty years ago by Martin

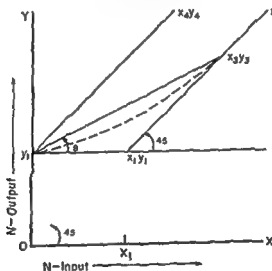


FIG 5. Mathematical expression of the theory of nitrogen balance. Adapted from Martin and Robison (1922)

and Robison (1922). This relates the nutritive value of dietary protein to changes in nitrogen balance with classic lucidity.

Let  $y_1$  be the output on a nitrogen free diet of adequate fuel value; then  $y_1$  is equal to the nitrogen minimum. Suppose that an ideal protein (biological value = 100) is fed in gradually increasing amounts and is utilized without waste. So long as the intake ( $x$ ) remains lower than  $y_1$ , the output will remain constant and equal to  $y_1$ , since the food protein

gives an equal amount of body protein. When the nitrogen input equals the nitrogen output ( $x_1 = y_1$ ) the body will be in nitrogen equilibrium. This point ( $x_1, y_1$ ) will represent the minimal protein needs of the body. If now the intake of the ideal protein be further increased equilibrium will again result (unless the body is in a growing condition or has been previously starved of nitrogen) and its graph will follow the line to  $x_2, y_2$  at an angle of  $45^\circ$  to the axes.

With proteins of biological value (BV) less than 100 minimal nitrogen equilibrium will not be attained on an intake equal to  $y_1$  but on some greater amount defined by the point  $x_3, y_3$ . On all amounts less than this the output will exceed the input and the graph will follow some line joining the points  $y_1$  and  $x_3, y_3$ . Whether this line is straight or curved will depend on the conditions set out above viz: (a) uniform caloric economy with varying nitrogen intake (b) amino acid pattern of the nitrogen requirements of the body. If these conditions obtain the line  $y_1, x_3, y_3$  will be straight and its equation will be  $y = y_1 + x \tan \theta$  where  $y$  is the real output corresponding to any real intake  $x$  less than  $x_3$ . For higher values of  $x$  the graph will follow the line joining the points  $x_3, y_3$  and  $x, y$ .

In this frame of reference THOMAS'S formulas (1909) can be very simply expressed in terms of  $\theta$  thus

$$BV = 100 \times \frac{\text{Urine N in N free diet} + \text{N balance}}{\text{N intake}}$$

$$BV = 100 \times \frac{y_1 + (x - y)}{x}$$

becomes

$$BV = 100 \times \frac{y_1 + x - (y_1 + x \tan \theta)}{x}$$

or

$$BV = 100 \times (1 - \tan \theta)$$

If the above conditions do not obtain e.g. if certain of the amino acids are required for specific purposes which are distinct and can be separately satisfied the graph of a protein diet rich in these acids but poor in others would be a curved line such as the dotted line joining points  $y_1$  and  $x_3, y_3$ . This curvature would express the fact that a certain fraction of the body's needs could be satisfied by a smaller amount of this protein than would correspond with the amount required to obtain equilibrium. The angle  $\theta$  and the biological value would then vary for different values of  $x$ .

The graph of a protein ( $BV = 0$ ) unable by itself to satisfy any

portion of the body's nitrogen requirements would be a straight line  $y_1, x_1 y_1$ , parallel to the slope of  $x_2 y_2$ , since the nitrogen output would always be equal to the intake  $+ y_1$ . For this line  $\theta = 45^\circ$  and the equation  $y = x \tan \theta$  becomes  $y = y_1 + x$ , while the biological value =  $100 (1 - \tan 45^\circ) = 0$

## B EXPERIMENTAL DATA

The demonstration by Rose (1938) that animals could be grown and maintained on mixtures of amino acids as well as whole proteins has greatly expanded the scope of the above theorem. Recent studies have shown that it may be employed to advantage in estimating the human need for certain amino acids in terms of amino acid blood levels (Albanese *et al.*, 1958). Many experimental procedures based on corollaries of the nitrogen balance theorem have been devised for the measurement of the nutritive value of proteins in terms of biological value, protein minima and nitrogen balance index (Allison 1949 also this book.)

### 1 Animal Studies

Most of the problems of application of the theory stem from the fact that the validity of the data depends on having an absolute nitrogen minimum value ( $E_0$ ) (Lang and Ranke 1950). The dependence of the nitrogen minimum on the quantity and quality of protein ingested prior to the depletion period was demonstrated as early as 1866 by Voit. He fed a dog weighing 35 kg different quantities of meat and measured the effect on urea output during the subsequent starvation period (Fig. 6). By deducting the urea eliminated on the sixth day when the output became constant at 120 gm per day from that excreted on previous days of starvation he obtained the grams of urea derived from previous food. Bread fed in *ad libitum* quantities showed poor nutritional value by this criterion. In later experiments with hogs, McCollum and Steenbock (1912) showed that the minimum nitrogen expenditure on a protein free starch diet was considerably lowered by feeding zein instead of urea in the fore period.

Mitchell (1923-1924) described the determination of the biological value of a protein by a method based upon nitrogen balance data obtained under dietary conditions which provided sufficient carbohydrate and fat to satisfy the energy requirements so that the catabolic processes were prevented. The basic calculation of Mitchell's biological value is

$$\frac{\text{Retained food N}}{\text{Absorbed food N}} \times 100 = BV$$

Values for the two unknown functions of this equation are derived from the following considerations

Absorbed food N = Food N — fecal food N

Fecal food N = Fecal N — Metabolic N of feces

Retained food N = Absorbed food N — Excreted food N

Excreted food N = Urine N — Endogenous N or urine

The items "metabolic N of feces" and "endogenous N or urine" are obtained by measuring the nitrogen output of animals maintained on

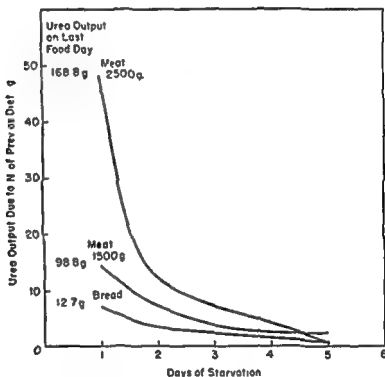


FIG. 6 Effect of previous diet on rate of nitrogen depletion of dogs (data of Voit 1866)

nitrogen free diet until a true nitrogen minimum level ( $y_1$  in Fig. 1) has been achieved. Unfortunately this value must be determined for each experiment since it varies not only from animal to animal but in the same animal at different times. The possibility that the distinction between exogenous and endogenous nitrogen is envisioned by Folin (1905) and derived from these calculations by Mitchell is no longer tenable according to the dynamic concept of protein metabolism in no way invalidates the results secured by the method. It is of more than passing interest to note that Mitchell also found that the biological value of proteins as determined by his procedure increased with a decrease

in protein content of the diet (Table V). This effect appears to be related in part to the biological value of the test proteins.

Melnick and Cowgill (1937) have demonstrated the linear relationship between nitrogen balance and per cent protein calories in the diet. On the basis of this relationship the nutritional value of proteins and amino acid mixtures can be estimated from the minimum amount of dietary nitrogen necessary to maintain nitrogen equilibrium. Allison (1949) and associates have further modified this technique so that the relationship of absorbed nitrogen and nitrogen balance are measured in

TABLE V  
RELATIONSHIP OF THE BIOLOGICAL VALUE ON THE AMOUNT OF PROTEIN IN THE DIET<sup>a</sup>

Protein	Biological Value	
	5% Protein	10% Protein
Milk	93	85
Oats	79	65
Corn	72	60
Potatoes	69	67

<sup>a</sup> Mitchell and Hamilton (1929)

the region of negative or slightly positive balance. The slope of the line obtained by a plot of these values, which are functions of nitrogen output and input given in Fig. 5, yields the *nitrogen balance index* of the foodstuff. This is equivalent to a function of the angle  $\theta^\circ$  (Fig. 5). It should be recognized that although these variants do not add anything essentially new to the theory of measuring biological value they do simplify visualization of the processes, especially in catabolic states.

## 2 Human Studies

The difficulties encountered in determining biological values or nitrogen balance indexes in experimental animals are also faced to an even greater degree in studies with humans. Hoffman and McNeil (1949) applied the nitrogen balance index procedure to human studies and found the following disadvantages: (a) the need of a long and difficult balance study, (b) irreducible error of the determination of the nitrogen minimum and (c) difficulty of securing data having a linear relationship.

These difficulties have long been known. Measurements of the minimum endogenous nitrogen metabolism of man made by the early workers have been cited by Millard Smith (1926). The data in Table VI has been taken mainly from his paper and from the recent findings of Bassett (1945). Attention is called to the variations in the nitrogen per kilogram values. Bassett's experiments on the effect of testosterone on the nitrogen minimum suggests that the hormonal state of the indi-

vidual may exert a more profound influence on the minimum nitrogen metabolism than has hitherto been realized

Apart from the nutritional history and hereditary factors of the organism it has been found that while the endogenous nitrogen per unit of body weight tends definitely to decrease with increasing weight and age the endogenous nitrogen is constant when related to basal metabolism (Terroine and Sorg Matter 1920 1928) This correlation seems to prevail not only for man but also for pigs cattle sheep dogs, and rats and may be due in part to hormonal or enzyme factors

TABLE VI  
MINIMUM EXCRETION OF NITROGEN IN URINE OF ADULT HUMANS\*

Investigator	Total urine nitrogen (gm)	Nitrogen per kilogram (gm)	Day of experiment
Folin	2.60	0.0406	12
Klemperer	2.51	0.0395	8
Thomas	2.98	0.0391	19
Graham and Foulton	2.25	0.0366	9
Robinson	1.99	0.0344	11
Klerker	2.01	0.0319	6
Siven	1.84	0.0317	7
Smith	1.58	0.0242	24
Bassett	2.03	0.0372	41 to 45
Bassett	1.39	0.0278	51 to 55 (Testosterone propionate)

\* Albanese (1950)

Murlin *et al* (1946) have also measured the excretion of nitrogen by man fed a protein free diet with the aim to determine biological values of proteins. They too found that the minimum nitrogen expenditure varied in man with (a) level of protein in the pre experimental diets (b) the position of the nonprotein period in the series of periods (c) the nature of the protein (supporting protein) immediately preceding the protein free period and its level of intake and (d) conditions antecedent to the supporting proteins which could influence the nitrogen debt to the beginning of the protein free period. In the face of such difficulties these investigators were able to ascertain that the biological value of mixtures of amino acids was always 10-40% lower than the natural proteins.

The adaptive mechanisms of the body serve to further confuse efforts at a quantitation of the protein needs by the nitrogen balance method. As long ago as 1900 Siven showed that nitrogen equilibrium could be maintained at a lower level than that ordinarily occurring in starvation.



In this experiment, which was divided into five periods of about a week each, a healthy man, weighing 60 kg, who normally ate a mixed diet containing 16 gm of nitrogen was given a diet containing 12.7, 10.4, 8.7, 6.3, and 4.5 gm of nitrogen rich in carbohydrate and yielding 2444 calories per day, in each successive period. Nitrogen equilibrium was established at all five levels of nutrition, albeit 3 and 4 days, respectively were required to achieve it at the lowest nitrogen intake levels. In a subsequent experiment on himself Siven, who was 31 years old and weighed 65 kg, reported that he attained nitrogen balance on an intake of 4 gm of protein nitrogen and 2717 calories (1901). Petren (1924) maintained a diabetic patient in nitrogen balance for a month or more

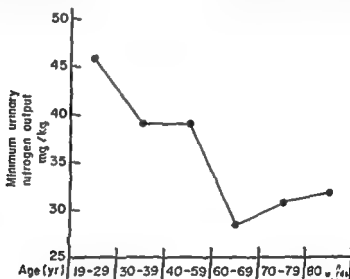


Fig 7 Effect of age on minimum urinary nitrogen output of man. Adapted from Schulze (1955)

on a diet containing 4 gm of protein nitrogen. Chittenden (1904) found that nitrogen equilibrium can be maintained on diets providing but 7.19 gm nitrogen per day and only 21.3 calories per kilogram. It would appear that amino acid and protein needs indicated by these low levels of nitrogen or caloric intake, are of little or no value as measures of dietary requirements for optimal health (Terroine 1957).

Schulze (1955) has collected and graphically summarized the available data on the effect of advancing years on the endogenous nitrogen output of man (Fig 7). Adaptation of older subjects to protein restriction has led to further discrepancies between the results of various investigators. To test this mechanism Schulze compared the nutritive values of wheat and milk proteins in elderly patients. For the first 10 days they were kept on a nitrogen free diet then placed for 10 days on a diet which was adequate in protein intake. The physiological net

utilization of milk protein proved to be higher in the older than in the young patients. On the other hand the net utilization of wheat proteins was much lower in the older age group than in the young group (Fig 8). These observations led Schulze to the conclusion that the minimal nitrogen expenditure in the aged decreases in proportion to the basal metabolic rate and that the phenomenon was not due to impairment of intestinal absorption of proteins in the elderly.

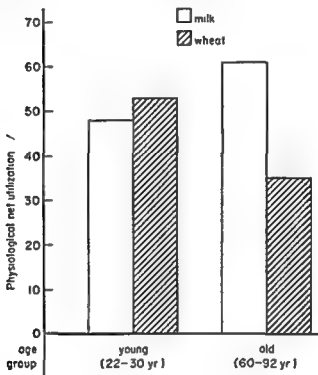


FIG. 8 Effect of age on human utilization of milk and wheat proteins

### C DEPLETION STATES

In this connection it should be noted that Allison (1949) has found nitrogen balance indexes of proteins to increase as the animal is depleted in proteins. This increase is illustrated by the data recorded in Table VII which also show that the effect of depletion is more marked on the indexes of the poorer proteins. Thus when there is greater need more nitrogen is retained and conserved in the body of the animal. Silber *et al* (1946) also found that depletion in proteins reduced the excretion of amino nitrogen following intravenous administration of amino acid mixtures.

It has long been known that in the anabolic phase of convalescence adults can store body proteins at a rate which is inversely proportional

TABLE VII  
NITROGEN BALANCE INDEXES IN NORMAL AND PROTEIN DEPLETED DOGS\*

Protein source	Nitrogen balance index	
	Normal	Depleted
Casein; whey	0.96	1.2
Casein	0.80	0.93
Casein hydrolyzate	0.80	0.92
Casein + gluten	0.44	0.70
Rotem	0.39	0.73

\* From Allison (1949)

the existing depletion (Forsyth *et al.*, 1955). The relation of the return of nitrogen balance and body weight to the norm is shown diagrammatically in Fig. 9. Other things being equal, it was noted in our laboratory that if the self-selected dietary was *suboptimal* (National Research Council, 1953) in terms of total calorie or protein content, normal nitrogen balance for the age of the subject was achieved slowly by decrements of an existing negative balance. On the other hand, if the self-selected diet was *optimal* in terms of total calorie or protein content, normal nitrogen balance was achieved quickly by decrements of the existing positive nitrogen balance.

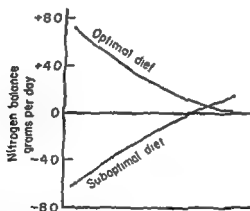


FIG. 9. Normal nitrogen balance recovery of convalescent adults consuming suboptimal and optimal self-selected diets.

In depletion states the need for a greater than normal allowance of calories and protein has been amply documented by Pollack and alpern (1952). Many evidences both clinical and biochemical, of a range from a suboptimal to an improved diet have been found to occur in undernourished adult convalescents receiving daily for 21 days a dietary supplement containing approximately 35 gm of milk proteins and 650 calories (Higgon and Albanese 1957). These investigations revealed that in depleted individuals (70-80% S) with negative nitrogen

balance, administration of the milk protein supplement was initially associated with a marked shift to a repletion level of positive nitrogen balance (Fig 10). With continuation of the supplement positive nitrogen balance fell to the normal range for healthy adults ( $+10 \pm 0.5$  gm N per day). Preliminary studies have revealed that excellent accord

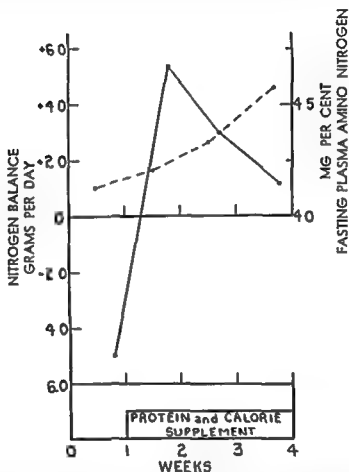


FIG 10 Representative nitrogen balance and fasting plasma amino nitrogen changes observed in underweight adult convalescents receiving a dietary supplement containing milk proteins 36 gm carbohydrate 64 gm and fats 28 gm. The average daily intake on a self selection basis was approximately 2000 calories and 65 gm of protein per day. Nitrogen balance is represented by the solid line and fasting plasma amino nitrogen by the broken line.

with the metabolic nitrogen patterns shown in Fig 10 also characterizes the nutritional improvement found to occur in adult convalescents receiving daily lysine supplements (600-900 mg) for periods of 1 or 2 weeks (Albanese *et al* 1957).

Unfortunately, the physiological decrease in positive nitrogen bal

ance, which may often be revealed by detailed studies of this kind, be misinterpreted to indicate an untoward effect of the test or supplements. Failure to realize the dynamic nature of these processes has led many a well intentioned investigator to cause metabolic stress in study subjects by excessive administration of protein or other nutrients for the purpose of enhancing an existing low but biologically optimal positive nitrogen balance. It seems evident from these considerations that although the nitrogen balance method constitutes a useful tool for the detection of protein or specific amino acid deficiencies which induce negative balances, it is less than an ideal procedure for the determination of protein stores and quality in undernourished subjects with substandard positive nitrogen balances. Indeed, it is this possible interpretative confusion of nitrogen balance data in a continuum of nutritional states of convalescents which led us to the use of other methods as criteria of protein metabolism.

The foregoing examples may be regarded as a sampling of the available evidences of the operation of Liebig's law of the minimum (1855). As noted above, this phenomenon makes possible the maintenance of positive nitrogen balances at planes of nutrition far below which human experience and history show to be necessary for optimum nutrition. It is obviously difficult for many to accept doubts as to the validity of nitrogen balance data obtained with low protein intake on the grounds of nutritional history. Dr McCollum's recent book, *History of Nutrition* (1957), reveals that the battle of parsimony versus generosity in nutrition has been fought with unlagging fervor since the days of the Venetian gentleman, Luigi Comaro (1467-1566).

The ultimate benefits of each of these nutritional attitudes have been questioned. Debate on the point can be long and futile. The answers lie in the future. Dr E. V. McCollum's comment (1939) on this subject is worthy of note. In the United States for about three hundred years we have been trying an experiment in human nutrition on a nationwide scale with a dietary which is of a kind which no people in the world ever tried to live upon before. There is no way in which the results of such an experiment can be foretold. We do know that our way of life has in the short span of three or four centuries produced a nation which is the envy and desired haven of many people throughout the world.

#### D. SUMMARY

The foregoing discussion points up the view that the measurement of nitrogen balance per se is not a sound or infallible criterion of protein nutrition. Unfortunately, the serious shortcomings of this measurement are not generally recognized. However, they are well known to

who have employed this tool in metabolic studies on experimental animals or human subjects. Interpretations of nitrogen balance data are especially fraught with imponderable hazards when derived from tests on spontaneously or artificially depleted organisms. This consideration applies in particular to experiments designed to determine protein or amino acid needs by procedures involving prior limited intake of the test nutrient. Improvements in nitrogen balance obtained by stepwise additions of the limiting nutrient give a measure of the need in a specifically depleted organism. It is not, as has been suggested by some, a measure of the test nutrient requirement for normal healthy organisms.

## IV OTHER METHODS

### A CREATININE EXCRETION

Folin (1905) as a result of his classic studies on protein metabolism, came to the conclusion that the daily output of creatinine of a given person is more or less constant, is influenced by the body weight and is independent of a diet which does not contain creatine or creatinine. At that time he introduced the term creatinine coefficient which is defined as the amount in milligrams of creatinine or creatinine nitrogen excreted per kilogram of body weight. Also since the excretion of creatinine is independent of the diet it was considered to represent the "endogenous metabolism" of the body as contrasted to the excretion of urea which represents the "exogenous metabolism". In Schaffer's opinion (1908) the creatinine output represented only a special phase of the endogenous metabolism which took place largely if not wholly in the muscles. Shaffer and later Myers and Fine (1913), and Hahn and Meyer (1928) adduced evidence that muscle creatine is the precursor of urinary creatinine. This transformation was proved conclusively by Bloch and Schoenheimer (1939) and Block *et al* (1941) by isotope tracer evidence which also showed that creatinine was the only normal urinary constituent containing any significant amount of body creatine nitrogen. Insofar as the biosynthesis of creatine is concerned it is now well established that three amino acids are the precursors (Schoenheimer 1942). The fatty acid chain is derived from glycine, the guanidine nucleus from arginine, and the glycoylamine thus formed is methylated by transfer of the labile methyl group from methionine.

#### 1 Children

The creatinine coefficient has been variously interpreted as proportional to or an index of (a) the amount of active protoplasmic tissue in the body by Folin (1905), (b) the muscular mass and efficiency of the individual by Schaffer (1908). In infants where the muscular mass

dominates the endogenous metabolism, the excretion of creatinine has been found by Catherwood and Stearns (1937) to be a function of weight with a correlation of 0.9. The correlation coefficient for creatinine excretion to body weight is about 0.8. At birth the creatinine output averages about 10 mg per kilogram. Breast fed infants remained at this level throughout the first year of life, whereas babies fed a formula of undiluted cow's milk showed a mean value of 12.5 mg of creatinine output per kilogram. The amount of musculature very quickly rises to a maximum in the infants given a higher protein diet.

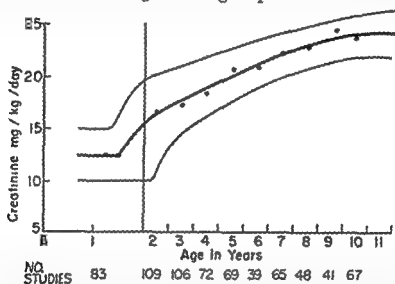


FIG. 11. Mean creatinine per kilogram for boys of each age group studied. The fine lines represent standard deviation for the given age. This figure indicates relative growth of the skeletal musculature in relation to total body growth (Stearns 1958).

Macy (1942) found that the preformed creatinine output of growing children rises concomitantly with increase in body weight in such a way that young children may show a creatinine coefficient comparable to that established in normal adults. The data in Fig. 11 relates daily creatinine excretion to body weight for children (Stearns, 1956). In children whose weight and height are within normal range, but whose nutrition has been somewhat substandard, except for calories, there are somewhat lower creatinine values. Apparently muscular development in children is dependent in a large part on the amount of available dietary protein. Stearns feels that the daily creatinine excretion is a very close measure of the total skeletal musculature of the child, and that the protein intake, permitting the quantity of muscle characteristic for any age, closely approximates the protein requirement for that age level.

Talbot (1936) proved that urinary creatinine excretion is directly related to basal metabolism in children. This relationship was amply

confirmed by Macy (1942) Talbot and co workers (1939) have also presented new creatinine standards for basal metabolism and clinical application for children. These data show that the creatinine standard is as accurate as weight standards for children who are normal, and suggest that it is of greater accuracy for those who are abnormal especially the obese because creatinine excretion is an index of muscle weight.

## 2. Adults

According to Hunter (1928), the creatinine coefficient for males varies from 20 to 26 and for females from 14 to 22. Hodgson and Lewis

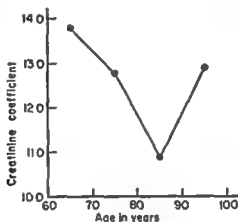


FIG. 12. Effect of age on creatinine coefficient. Data are based on 259 consecutive measurements on 22 individuals over a 7 year period.

(1926) consider the difference in creatinine coefficients of males and females to be related to differences in muscular development rather than sex differences since they found women with unusual muscular development to have creatinine coefficients comparable to those of males. Corpulent or even moderately corpulent subjects yield less creatinine in the urine per unit of body weight than do lean ones. Moderately corpulent persons eliminate daily 20 mg of creatinine per kilogram of body weight while lean ones yield about 25 mg per kilogram. McCluggage and associates (1931) found that upon weight reduction through dietary control the creatinine excretion of obese persons remained constant. These observations on adults are in good agreement with those of Talbot on children.

Serial measurements done in our laboratory on a group of women (65-95 years of age) show that the creatinine coefficient falls with age to the levels found in infants (Fig. 12). In the aged this fall in creatinine coefficient parallels the drop in basal metabolic rate.



### 3 Creatine

In 1905, Fohn stated that creatine is absent in the urine of the normal male adult. Despite mounting evidence to the contrary, this statement continues to appear in many textbooks and reviews. Albanese and Wangerin (1944) and Wilder and Morgulis (1952), using improved methods of analysis, have shown that normal adult males regularly excrete small amounts of creatine (60-150 mg per day). Although the great majority of females excrete about twice the creatine output of males, in about one-fifth of the females the creatine excretion is similar to that for the males. The report of Milhorat and Wolff (1937) has shown that in progressive muscular dystrophy there occurs with declining muscle function a proportionate decrease in creatinine and increase in creatine output. These changes are not specific since augmented creatinuria occurs in starvation and hypothyroidism. From these observations it does not appear that creatine output is as useful an index of protein nutrition as the creatinine excretion.

### B PLASMA AMINO NITROGEN

The free amino acids of the blood arise from absorption, synthesis and tissue breakdown. These processes continually add and remove amino acids from the blood. Consequently, the amino acid level of the blood, like levels of blood sugar and other blood constituents, represents the balance between the rates of addition and removal. The metabolic and clinical significance of free amino acids of the blood has long been studied. The relation of aminoacidemias to various disease entities was reviewed by Re (1940). Within the last decade, application of the newer chemical and chromatographic methods suggests that plasma amino nitrogen levels may provide a useful criterion of nutritional status and of protein nutrition in particular. Some of the significant contributions supporting this biochemical concept include the report by Man and his co-workers (1946) which showed that low fasting plasma levels of amino nitrogen (below 4 mg %) were invariably associated with poor nutritional status of preoperative patients. The finding of Bonsnes (1947), that the plasma amino acid level of pregnant women is significantly lower than that of nonpregnant women, has been amply confirmed by Clemetson and Churchman (1955). Further, the studies of Everson and Fritschel (1951) revealed that a group of postsurgical undernourished patients had significantly lower plasma levels for each of the individual essential amino acids. These investigators also demonstrated that decreased levels of total and individual essential amino acids (especially lysine) may provide an earlier biochemical index of malnutrition than the measurement of blood albumin levels.

Levenson and Rosen (1954) have observed that in the plasma of severely injured soldiers the individual free amino acids proline threonine histidine and glycine stayed near normal concentrations lysine leucine isoleucine valine tyrosine alanine and taurine rose sharply on the third or fourth day after injury (a period of high catabolism) and fell below the norm about the tenth day. In addition large quantities of an amino acid conjugate appeared characteristically in the plasma of patients with injury or renal dysfunction. The striking central fact was the quantitative and qualitative variability of the composition of this fraction among patients. This conjugate contained large quantities of glycine and glutamic acid but was lacking or poor in lysine phenylalanine and valine. These investigators feel that inadequate food intake was one of the important factors in the pathogenesis of the malnutrition observed in these severely wounded soldiers.

In order to determine the biochemical significance of plasma free amino nitrogen levels Albares and co-workers (1958) attempted to correlate this measurement with other criteria generally employed for the evaluation of nutritional status in population or clinical groups. In these studies despite some obvious pitfalls the observed body weight deviations of our subjects as per cent of the standards (% S) indicated by the Metropolitan Life Insurance Company Tables of 1951 was correlated with fasting plasma amino nitrogen (PAN) levels. The composite curve (Fig. 13) for the adult subjects tested shows that the average PAN level falls sharply to approximately 4 mg % with the decline of body weight to the 80-90% S level and remains about 4 mg % from the 80-60% S range. In children however, the PAN level falls to an average 3.64 mg % in the body weight range 70-80% S. It may be biochemically significant that the PAN values for children in the normal weight range are slightly higher than that of adults in the same % S area and falls to a lower level in the nutritionally substandard states.

It will also be noted in Fig. 13 that a somewhat higher fasting PAN level occurs in the 60-70% S than in the 70-80% S range. This finding tentatively until more data are accumulated suggests that the higher PAN levels found in the 60-70% S range are probably the result of high catabolic rates prevailing at this degree of overt malnutrition. Further support for this view is provided by two cachectic individuals (terminal cancer) whose body weights were in the 50-60% S range and had fasting PAN levels above 5 mg %—a value significantly higher than subjects in the 90-100% S range.

Studies on the relationship of PAN levels to body weight change and food intake of 14 infants (2-22 months of age) recovering from a variety of diseases and surgical procedures have recently been completed in

our laboratory. The data on four of these studies, which are quite typical of the rest, are shown graphically in Fig. 14. It is at once apparent that good correlation prevails between PAN levels and body weight changes. Furthermore, PAN and body weight changes reflect calorie and protein intake with a remarkable accuracy. In general, it can be stated that protein intakes of less than 50 gm per kilogram were associated with a downward shift in these two criteria of nutritional status. In one

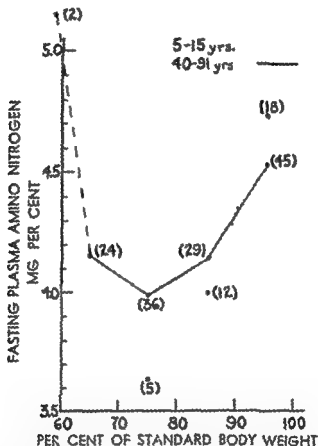


FIG. 13. Relation of fasting plasma amino nitrogen levels to per cent deviation from standard body weight (% S). Values in parentheses indicate number of subjects tested.

instance R. E. (week 5), a fall in PAN and body weight change accompanied an increase in the per cent of protein calories.

Further attempts to evaluate the significance of PAN levels as criteria of protein nutrition led to investigations of the effect of protein loads on the amino nitrogen content of the blood. The results of this study are shown graphically in Fig. 15. The ordinate, PAN index of protein utilization is empirically defined here as the difference in PAN content between the fasting sample and that found 60 minutes after oral administration of the protein load ( $\Delta A_{60}$ ) times 100, divided by the fasting

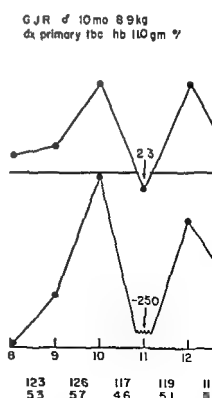
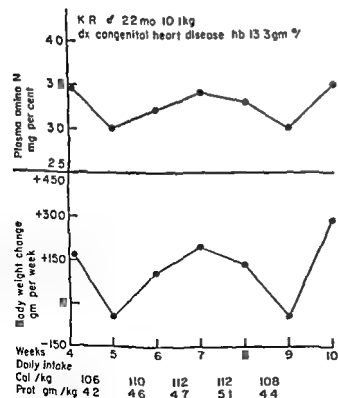
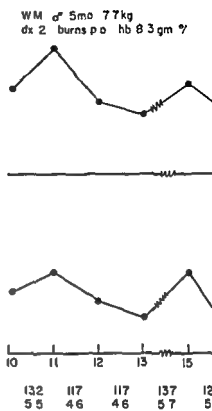
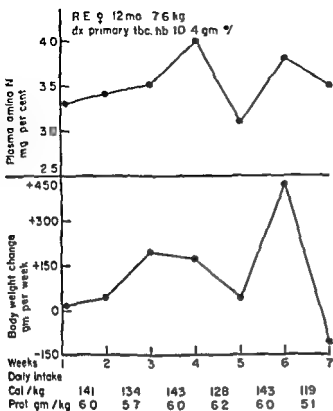


Fig. 14. Plasma amino N and body weight change in four rats.

amino nitrogen ( $A_F$ ) As might be expected from the biological principle of diminishing increments (Brody, 1945), the regression line of our data clearly shows the tendency for a decline in utilization of dietary proteins with improved nutrition, measured in this instance in terms of fasting PAN concentrations

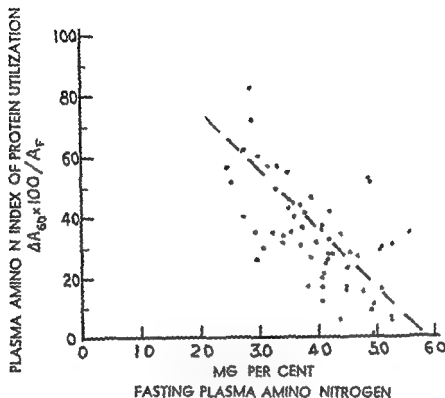


FIG 15 Fasting plasma amino nitrogen levels and utilization of protein load in adults 40-77 years of age

### C SPECIFIC PLASMA AMINO ACIDS

Rather convincing evidence is on hand which shows that the pattern of amino acids in the diet markedly influences the level of some free amino acids in the blood. In poultry, Charkey and associates (1950, 1953), as well as Almquist (1954) have observed good correlation between amino acid levels in chick blood and composition of dietary proteins. Fisher (1957), using amino acid mixtures, found that changes in blood levels of some amino acids serve as good indexes of the dietary need of specific amino acids.

Denton and Elvehjem (1954a, b) reported that the portal and radial vein concentrations of individual essential amino acids in dogs were rapidly increased in proportion to the levels supplied by the test proteins, casein and beef. In the case of the imbalanced protein, zein which lacks lysine and tryptophan, an initial drop occurred in the total free

amino acid level. This was followed by gradual increases in plasma concentration of several amino acids particularly isoleucine, leucine and phenylalanine which are abundant in zein. Lysine levels remained depressed. Tryptophan concentrations were well maintained on the zein diet as well as on protein free meals. Albanese and Orto (1955) have noted an accord between lysine levels in the diet and free lysine level in the blood of infants.

The aggregate of the foregoing observations have led the author to speculate on the possible interpolation of fasting plasma levels of indi-

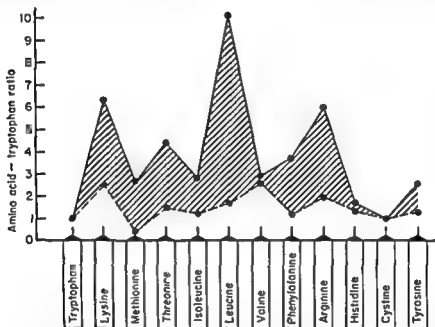


FIG 16. Amino acid pattern differences of muscle proteins and fasting plasma amino acids. The hatched areas represent the specific amino acid deficit. Mammalian muscle proteins ———. Fasting plasma amino acids — — —.

vidual amino acids and their dietary requirements as indicated by the carcass theory discussed by Mitchell (1959). Pattern calculations from the available data on free amino acids in fasting human plasma and the average amino acid content of mammalian tissues (Steele *et al.* 1950) were done. The pattern deficit of fasting plasma amino acid levels with reference to the amino acid contour needed for tissue synthesis is shown in Fig 16. Further calculations on the amino acid contribution of various foods to these deficits show that meats provide an optimal corrective pattern, cereals provide good corrections with the exception of lysine and possibly threonine. In this frame of reference the amino acid pattern of diets of low economic groups in the United States was estimated

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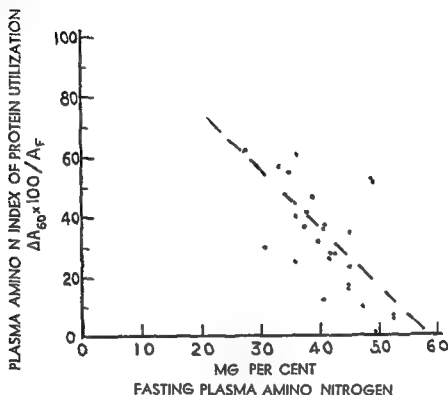


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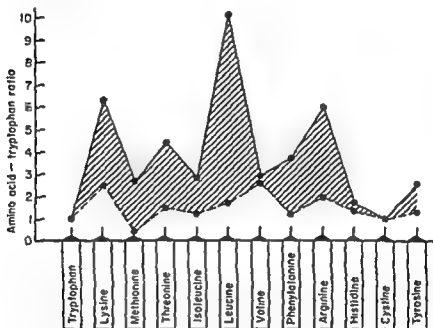


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to be poor in lysine and threonine. Analyses of food consumption of convalescents and the aged indicate that their diets may be limiting in lysine, threonine, isoleucine and valine.

Preliminary investigations by Albanese *et al* (1959a, b) indicate that the nutritional adequacy of some breakfast patterns indulged in by young children may be evaluated from differences in plasma amino acid levels measured immediately before, and 1 hour after the test meals.

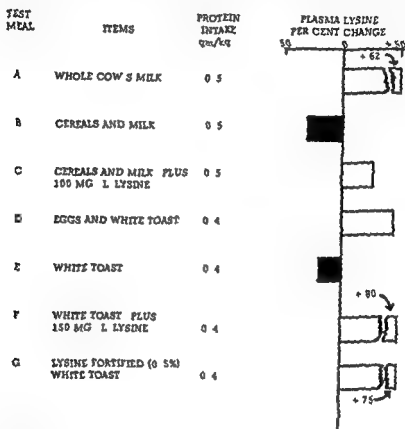


FIG 17 Summary of effects of test meals on plasma lysine levels in young children (average results)

These meals supplied proteins and calories (per kilogram of body weight) in amounts normally consumed by young children, and were tested on some 30 normal healthy children 2.5 to 12 years of age and 90-122% of standard body weight. The results on the availability of lysine from the foods assayed are summarized in Fig 17. Briefly, these experiments show that *ad libitum* consumption of fresh cow's milk caused an increase in plasma lysine levels. Ingestion of typical cereal milk breakfasts induced a decrease in plasma lysine which could be overcome by small additions of L-lysine to the fruit juice. Similar studies showed that egg and white toast breakfasts caused an increase in plasma lysine levels. Toast alone induced plasma lysine deficits which could be cor-

ted by additions of lysine to the fruit juice, or to the bread prior to  
ing

The studies of Longenecker and House (1958) on adult dogs dis-  
se that plasma amino acid changes indicate that lysine is the first  
ing amino acid for wheat gluten tryptophan for gelatin, and ar-  
ine for casein

#### D URINARY AMINO ACIDS

There are considerable variations in the amounts of particular amino  
ds excreted by different normal individuals (Steele *et al.*, 1950 Barry,  
53) The causes of these variations are not entirely understood and  
dently differ from amino acid to amino acid It is known however,  
t differences in diet, genetic differences between individual people,  
d physiological changes such as pregnancy may contribute to such  
iations (Williams, 1959)

In general it has been found that neither the total quantity nor the  
tribution of the amino acids in normal urine can be correlated closely  
th the dietary intake of protein (Moore and Stein, 1951 Nassett and  
lley, 1952 Kirsner *et al.* 1949) Ten to fifteenfold increases in the  
etary protein give rise in most cases to no more than a two or three-  
d increase in the excretion of individual amino acids The one excep-  
n to this is L-methylhistidine The excretion of this substance is  
sely related to the amount of meat in the diet (Stein *et al.* 1954  
atta and Harris 1951) and it is probable that this histidine derivative  
largely derived from the dipeptide anserine often present in quite  
ge quantities in muscle However apart from L-methylhistidine it is  
ar that the variation encountered between different individuals in  
ount and pattern of amino acid excretion is greater than can be  
counted for in terms of dietary differences Even in a sample of urine  
lected 3 hours after feeding 50 gm of casein Stein and co workers  
954) found the usual amino acid pattern and the quantitative values  
re no higher than those they had found in other individuals on ordi-  
ry diets However Holt and Albanese (1944) have reported a  
nificant decrease in the tryptophan output of normal young males  
untained on a semisynthetic diet in which the principal nitrogen  
diet was comprised of a tryptophan deficient casein hydrolyzate The  
cretion of  $\beta$ -aminoisobutyric acid appears under ordinary conditions  
be little influenced by dietary variations though it has been found  
at complete fasting for 2-3 days may lead to an increased excretion  
andler and Pare, 1954) It is possible that observed differences in  
cretion of this substance may be due to genetically determined differ-  
ces in the renal tubular capacity for its reabsorption (Harris 1955)

During pregnancy there is an increase in the excretion of several urinary amino acids, histidine and threonine being the ones most markedly affected (Wallraff *et al* 1950). Threonine output increases steadily throughout gestation, whereas histidine reaches a maximum at about 4 months and thereafter remains at more or less the same level until term (Ruttinger *et al*, 1954). During lactation the excretion levels fall rapidly often to values below those found in the nonpregnant state.

### E ENZYME LEVELS

Under conditions of a nitrogen limiting dietary (specific or total) it is conceivable that loss of body protein involved mainly in structural functions of the organism would not be so detrimental were it not for

TABLE VIII  
COMPARISON OF APPROXIMATE PERCENTAGE OF AMINO ACIDS IN SOME ENZYMES AND AVERAGE MAMMALIAN TISSUES

Amino acid	yellow enzyme	Pepsin <sup>a</sup>	Chymotrypsinogen <sup>a</sup>	Ribonuclease <sup>a</sup>	Cytochrome <sup>b</sup>	Aldolase <sup>c</sup>	Average mammalian tissues
Lysine	13.6	0.9	8.0	10.4	22	9.5	8.0
Arginine	8.1	1.0	16.2	5.2	2	6.3	6.4
Histidine	2.8	0.9	1.2	4.2	3	4.2	3.0
Tyrosine	7.7	8.5	3.0	7.9	4.5	5.3	3.1
Tryptophan	4.8	2.4	5.6	0	1	2.3	1.3
Phenylalanine	5.6	6.4	3.6	3.6		3.1	4.0
Half cystine	1.0	1.6	3.3	6.5	4	1.1	2.2
Methionine		1.7	1.2	4.4	2	1.2	2.4
Serine		12.2	11.4	12.0		6.6	5.7
Threonine		9.6	11.4	9.0		7.1	4.7
Valine		7.1	10.1	7.3		7.4	5.2
Leucine		10.4	10.4	0		11.5	7.4
Isoleucine		10.8	5.7	3.1		7.9	5.1

<sup>a</sup> Tristram (1949)

<sup>b</sup> Theorell (1941) Based on number of amino acids per molecule of cytochrome c containing a total of 96 amino acids

<sup>c</sup> Velick and Ronzoni (1948)

the fact that tissue enzymes which are also proteins are built from the same essential amino acids that are utilized in building body proteins. It is apparent from the data contained in Table VIII that the most serious effects of specific amino acid or total protein deficiencies may arise as a result of their limiting effects upon the biosynthesis of enzyme systems in the animal body.

During the past ten years much experimental evidence has been reported relating levels of dietary proteins to tissue enzyme content. In

1948 Miller found that inanition in rats caused a loss of liver catalase alkaline phosphatase xanthine dehydrogenase and cathepsin which paralleled or exceeded the loss of liver protein. Potter and Klug (1947) had previously observed that liver octonate and succinate oxidases were depressed in rats fed only 6-10% protein diets. Lightbody and Kleinman (1939) reported that liver arginase activity of rats fed a 6% milk protein diet was about one half that of rats fed a 25% milk protein diet. Seifter *et al* (1948) made the interesting observation that both liver arginase and  $\alpha$ -amino acid oxidase are lost more rapidly than liver nitrogen in rats fed a nonprotein diet. In 1949 Williams and co-workers observed changes in dietary protein. The livers of animals fed a 14.6% casein ration plus 0.25% dietary methionine had twice the xanthine oxidase of those animals which did not receive the methionine supplement. With a lysine deficiency a 50% decrease in liver xanthine oxidase activity was associated with marked depressions in succinic and liver choline oxidases.

This method of study has also been applied to a number of different proteins (Litwack *et al* 1953). In this list beef round appears to give the best xanthine oxidase response followed by casein and lactalbumin with gliadin running a poor fourth. Lysine fortified gliadin gave a much greater response. When both lysine and tryptophan were added the value of gliadin was brought almost to that of casein. The body weight response of the rats so studied paralleled very closely their liver xanthine oxidase activity.

Van Pilsum *et al* (1957) measured the *in vitro* activities of kidney  $\alpha$ -amino acid oxidase and liver arginase, aconitase and xanthine oxidase of rats fed complete amino acid diets and diets deficient in either tryptophan, isoleucine or phenylalanine. They found that the activities per milligram of tissue nitrogen of arginase, aconitase and  $\alpha$ -amino acid oxidase did not change in the deficient animals. However the activity of catalase and xanthine oxidase per liver decreased in the deficient animals. Comparison of these results with those obtained by the previously cited investigators employing low protein or protein free diets suggests that the influence of these deficiencies depended upon the relative priority of the synthetic systems for the amino acid pool or the amino acid contour of the enzyme system (Table VIII). Ross and Batt (1957) have further investigated the relationship between activity of hepatic enzyme and diet and also between hepatic enzyme activity and age. They found that diets containing 15-20% of casein altered the levels of enzyme activity from those of young rats to those of older rats while diets containing 5-10% of casein altered the levels of activity of

older rats to those of more youthful rats. This quantitative enzymatic adaptation would seem to permit arbitrary adjustment by dietary means of the activities of some enzymes from one age level to another (or from one nutritional state to another) without significant changes in body weight.

Although these enzyme techniques hold promise of offering a specific and easily performed method for the evaluation of dietary proteins in experimental animals, their direct application to humans presents difficulties. However, some indirect procedures for human studies embodying this very important principle have recently been described. Wroblewski (1958) has made some useful correlations between serum enzyme alterations and tissue metabolic activity in various disease states. Though the efforts of this group have been directed primarily to serum utamic oxalacetic transaminase (S GOT) changes in neoplastic entities, our data also show good correlation with nutritional state of the patients.

As might be expected, the enzyme diet relationship affects not only enzymes of intermediary metabolism but also the proteolytic enzymes of the latter category of enzymes may lead to gastrointestinal malabsorption. It has been shown by Melnick *et al* (1946) that the rate of enzymatic digestion of dietary proteins constitutes an important factor in nutrition and the relative biological value of various food proteins. Since the rate of enzymatic digestion is a function of the availability of proteolytic enzyme, the importance of the diet enzyme relationship to nutritional state is at once apparent. It has been amply demonstrated that exocrine insufficiency of the pancreas occurs in all degrees and in greater frequency than heretofore suspected (Pollak, 1957). The enzyme content of exocrine pancreatic secretion by nature of its amino acid content is probably very sensitively influenced by the dietary protein intake. Normally the daily quantities of enzyme protein synthesis are enormous and the rate of uptake of labeled amino acids by the acinar cells is very rapid (Friedberg *et al*, 1948). It is not surprising then that protein malnutrition has been shown to suppress pancreatic enzyme formation (Trowell *et al* 1954; Jackson and Linder 1953). Extreme protein lack as in kwashiorkor eventually results in necrosis and atrophy of the acinar cells.

Since peptic digestion constitutes such an important determinant in the utilization of proteins, we recently undertook studies to quantitate the nutritional importance of this biochemical function in order to determine the effect of digestive enzyme administration on the absorption rate of milk proteins in terms of plasma amino nitrogen (PAN) change. Measurements were made on fasting subjects receiving on successive

days an oral protein load (0.2 gm protein per kilogram) with and without a mixture of pancreatin (300 mg) and pepsin (250 mg). The pattern of amino acid liberation was determined semi quantitatively by unidimensional chromatography. To date 13 children (5.5-12 years) and 60 adults (59-89 years) have been studied by this procedure. The children were healthy and normal and as might be expected showed no significant change in PAN index with enzyme administration. Results on the adults disclosed that enzyme administration significantly increased the PAN index of 19 subjects. These increases were most frequently associated with malnutrition and advanced age. Week long administration of the enzyme mixture with meals to 5 nutritionally substandard adults showed that the increases in PAN index were associated with increases in positive nitrogen balance. Aminograms revealed that positive responses in nitrogen balance generally included significant increases in essential amino acid content of the blood, especially methionine, threonine and lysine. Chromatographic analyses of *in vitro* enzyme milk protein digests indicated that the amino acid pattern changes of the blood in the oral tests could be ascribed to the enzyme supplement.

#### F METABOLIC RATES

Determination of the rate of oxygen consumption is also a useful criterion of protein nutrition because it measures the sum total and rate of all metabolic processes occurring in the body. The metabolic rate is regulated by endogenous and exogenous factors. The principal endogenous factors are age and sex (Lusk 1928). The effect of age is shown in Fig. 18. The difference between sexes is greatest on a percentage basis at the age of 19 where it is 13% greater for males. All the way from the age of 12-21 years the basal rate is some 10-12% less for females than for males (Albritton 1953). It is significant that the sex differences appear not only in the average rate of development but also in the nitrogen requirements at these ages.

Clinical use of calorimetry was presaged by Frederick Muller (1893) who noted that patients with Graves disease lost weight with marked nitrogen wastage in the face of diets adequate for normal nutrition. These findings were soon verified by Magnus Levy (1895) who ascertained the action of the thyroid in regulating the rate of combustion in the body. Although other factors are now known to affect the rate of heat production in the majority of cases variations in basal metabolism from the age sex norm can be safely interpreted as alterations in the function of the thyroid gland. Variations in metabolic rate are generally expressed in per cent of normal with  $\pm 15\%$  of the DuBois standards (1916) being considered as normal. Values from 40% below to 130%

older rats to those of more youthful rats. This quantitative enzymatic adaptation would seem to permit arbitrary adjustment by dietary means of the activities of some enzymes from one age level to another (or from one nutritional state to another) without significant changes in body weight.

Although these enzyme techniques hold promise of offering a specific and easily performed method for the evaluation of dietary proteins in experimental animals, their direct application to humans presents difficulties. However, some indirect procedures for human studies embodying this very important principle have recently been described. Wroblewski (1958) has made some useful correlations between serum enzyme alterations and tissue metabolic activity in various disease states. Though the efforts of this group have been directed primarily to serum glutamic oxalacetic transaminase (SGOT) changes in neoplastic entities, their data also show good correlation with nutritional state of their patients.

As might be expected, the enzyme diet relationship affects not only the enzymes of intermediary metabolism but also the proteolytic enzymes. Deficiencies in the latter category of enzymes may lead to gastrointestinal malabsorption. It has been shown by Melnick *et al* (1946) that the rate of enzymatic digestion of dietary proteins constitutes an important factor in nutrition and the relative biological value of various food proteins. Since the rate of enzymatic digestion is a function of the availability of proteolytic enzyme, the importance of the diet enzyme relationship to nutritional state is at once apparent. It has been amply demonstrated that exocrine insufficiency of the pancreas occurs in all degrees, and in greater frequency than heretofore suspected (Pollak, 1957). The enzyme content of exocrine pancreatic secretion by nature of its amino acid content, is probably very sensitively influenced by the dietary protein intake. Normally the daily quantities of enzyme protein synthesis are enormous and the rate of uptake of labeled amino acids by the acinar cells is very rapid (Friedberg *et al*, 1948). It is not surprising then that protein malnutrition has been shown to suppress pancreatic enzyme formation (Trowell *et al* 1954, Jackson and Linder 1953). Extreme protein lack as in kwashiorkor eventually results in necrosis and atrophy of the acinar cells.

Since peptic digestion constitutes such an important determinant in the utilization of proteins, we recently undertook studies to quantitate the nutritional importance of this biochemical function in order to determine the effect of digestive enzyme administration on the absorption rate of milk proteins in terms of plasma amino nitrogen (PAN) change. Measurements were made on fasting subjects receiving on successive

days on oral protein load (0.2 gm protein per kilogram) with and without a mixture of pancreatin (300 mg) and pepsin (250 mg). The pattern of amino acid liberation was determined semi quantitatively by unidimensional chromatography. To date 13 children (5.5-12 years) and 60 adults (59-89 years) have been studied by this procedure. The children were healthy and normal and, as might be expected, showed no significant change in PAN index with enzyme administration. Results on the adults disclosed that enzyme administration significantly increased the PAN index of 19 subjects. These increases were most frequently associated with malnutrition and advanced age. Week long administration of the enzyme mixture with meals to 5 nutritionally substandard adults showed that the increases in PAN index were associated with increases in positive nitrogen balance. Aminograms revealed that positive responses in nitrogen balance generally included significant increases in essential amino acid content of the blood especially methionine, threonine and lysine. Chromatographic analyses of *in vitro* enzyme milk protein digests indicated that the amino acid pattern changes of the blood in the oral tests could be ascribed to the enzyme supplement.

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above the norm may occur and are respectively indicative of hypothyroidism or hyperthyroidism. However, these disorders may arise as secondary effects of thyroid controlling substances of the adrenal or pituitary glands.

The greatest single exogenous factor which regulates the basal metabolic rate of the individual is the plane of nutrition (Brody, 1945). Regardless of how metabolism may be estimated, whether on the basis of weight or surface area, undernutrition or prolonged fasting in children

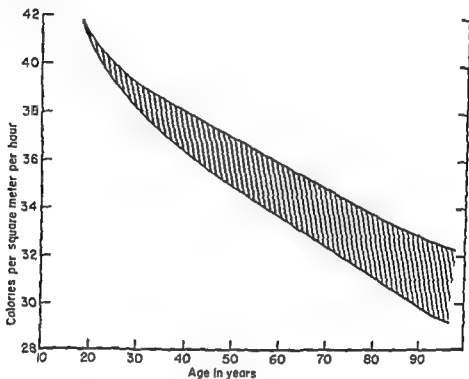


FIG. 18 Effect of age on metabolic rates. Idealized representation from the data recorded by Shock *et al* (1955)

results in a definite reduction of metabolic rate. The exhaustion of infectious fevers produces a state somewhat analogous to that of fasting. In older children there seems to be no definite relationship between the degree of underweight and the percentage increase in metabolism. In his fasting man, Benedict (1915) found that there appeared to be a specific reduction in metabolism coincident with undernutrition. This experience has since been abundantly confirmed. Overnutrition (obesity) arising from exogenous or endogenous factors is associated with low BMR in children and adults of both sexes.

More than ample evidence has been recorded by Brody to show that the 0.7 power of body weight is not only a good reference point for basal energy metabolism but also for basal protein metabolism or

endogenous nitrogen and neutral sulfur excretion. The creatinine excretion however varies not with the 0.7 power but more directly with unit weight. Calculations from the parallelism between basal energy metabolism and endogenous nitrogen excretion as functions of body weight show that a ratio of about 2 mg of endogenous nitrogen per calorie reveals for small or large animals. For the human Terroine and Sorg (1920-1928) reported a value of 2.32 mg nitrogen per calorie or total endogenous nitrogen excretion which included fecal nitrogen.

Determination of endogenous nitrogen excretion/calorie ratios during growth periods are complicated by the fact that maintenance of the test organism on a protein free diet results in a cessation of growth. One can only speak therefore of apparent endogenous nitrogen and basal metabolism during growth. It is interesting to note in this connection that Mitchell and Hamilton (1929) found that in growing rats the lowest nitrogen excretion is attained not on a protein free diet (0.5 mg nitrogen per grams air dried food) but on one containing 5-6 mg nitrogen, in the form of egg yolk per gram of air dried food. Since the apparent endogenous nitrogen level is influenced by many factors—dietary protein level and quality before and during the period of specific nitrogen turnover age and other factors—it can be readily understood that the observations on the value of endogenous nitrogen/calories may show wide discrepancies.

Lusk (1928) estimated that the maintenance energy requirement is between 11 and 15% above the starvation minimum. Maintenance being differentiated from productive living in that maintenance living does not involve the production of milk, eggs, wool, flesh, etc. or muscular work aside from that associated with carrying out the normal nonproductive life processes. The major maintenance energy expense is the basal energy metabolism. The "activity increment with respect to the basal metabolism has been reported to be 50-75% in humans under sedentary occupation" (Orr and Leitch 1937-1939).

The decline in total metabolism with increasing age is associated in part with declining body weight and in part with decreasing activity. Evidence bearing on the effect of age on the decreased metabolic rate and urinary nitrogen found in some of our studies is shown in Table IX. These data indicate that aging from 25 years to 80 years is associated with a 15% reduction in "active protoplasmic mass" (0 ml/min/kg). This reduction is accompanied by a 43% reduction in urinary nitrogen output.

The foregoing considerations have long led to the impression that measurements of metabolic rates with a careful interpretation of all the specified factors could serve as a measure of protein needs (Terroine

and Sorg Matter, 1920) This is particularly true in studies of the protein needs of aging adults Six year long serial metabolic measurements on 30 women (72-99 years) have shown us that resting  $O_2$  consumption per kilogram of body weight remains relatively constant for healthy

TABLE IX  
EFFECT OF AGE ON OXYGEN CONSUMPTION AND URINARY NITROGEN

Number of subjects	Age in years	Mean age in years	Average body weight (kg)	$O_2$ consumption (ml/min/kg)	Urinary nitrogen (mg/kg)
26	21-30	25	83	3.38	170
22	66-93	80	63	2.92	97

individuals 70 years of age and over The resting calorie per kilogram per hour need, however, varies inversely as the per cent of standard body weight (Fig 19) This correlation suggests that the active protoplasmic mass in the aged remains relatively constant through the range of 50-150% of standard

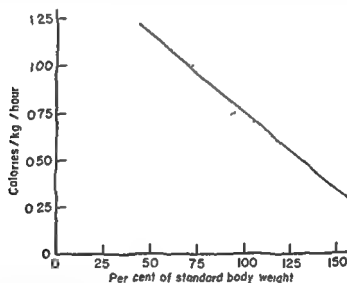


FIG 19 Relation of metabolic rate and per cent of standard body weight in women 72-99 years of age

During the course of the metabolic rate measurements, analyses of the dietary for total calorie and protein intake and of 24 hour urine collections for total nitrogen content were also done These data revealed that the average resting metabolism for these women, who were in normal positive nitrogen balance, was 1200 calories per day, and the average nitrogen output 6 mg per calorie The measured average daily calorie intake was 1560 which provided an average of 5.8 mg of nitrogen

per calorie. These calculations reveal that a good record existed between the protein/calorie ratio of the diet and that required by the metabolic pattern of our subjects. Furthermore it would appear that normal life activities of our subjects required but 360 calories per day (1560-1200) and 2.2 gm of protein nitrogen above the basal needs. These observations further disclose that the urinary nitrogen output of our subjects was approximately four times greater than the average endogenous nitrogen minimum of 20 mg of nitrogen per calorie (Schulze 1955). It would appear worthwhile to explore further the usefulness of metabolic rate measurements in establishing optimal dietary patterns, protein quality of diets and protein needs.

### G APPETITE

Osborne and Mendel (1918) in their rat feeding investigations with synthetic rations containing highly digestible constituents and possessing no pronounced flavors observed that there is a marked tendency for experimental animals to eat sparingly of deficient or nutritionally imbalanced diets and to increase food intake when balance of the dietary is restored. Subsequent reports of animal studies have amply confirmed this phenomenon especially with reference to the essential amino acids—particularly lysine (Rosenberg and Rohdenberg 1952).

In 1947 Albanese and co workers noted that the volume of a semi-synthetic diet consumed by infants was markedly reduced by the removal of tryptophan. This decrease in appetite which was apparently not due to an alteration in taste or odor disappeared overnight when dietary amino acid balance was restored by addition of the missing component—tryptophan.

In a recent publication (Albanese *et al* 1955) we mentioned that, when a lysine containing dietary supplement was given, an increase in appetite was noted in underweight infants who had a prior history of suboptimal food intake. The implications of this observation in terms of possible amino acid imbalances which may arise in the diets of infants under conditions of endogenous or exogenous nutritional stress prompted us to attempt a quantitation of the appetite stimulating effect of lysine supplements. To this end the daily dietary records were kept for 5 nutritionally substandard infants maintained on *ad libitum* feedings of the conventional evaporated milk formula for a pre lysine control period, a period on L lysine HCl and a post lysine control period. These diet records were then analyzed for average daily total calorie and protein intake during the lysine, and precontrol and postcontrol periods. The resulting data are shown graphically in Fig 20 and disclose that lysine fortification of the infant dietary was associated with an average 19%

increased consumption of milk, and an average of 13% increased intake of total calories. This improved nutriture was lost with the removal of lysine supplement from the infants' diets. More recently, this phenomenon has also been observed in undernourished older children and elderly convalescents. Since it is generally agreed that nutritionally better foods make for better appetites, the appetite response must be regarded as an overt physiological criterion of improved nutritional balance of the dietary.

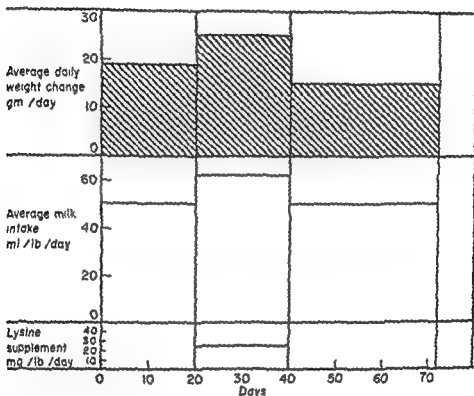


FIG. 20. Effect of lysine supplementation of evaporated milk formulas on intake and body weight change in five nutritionally substandard infants.

## H. SUMMARY

The foregoing discussion and consideration of supplementary criteria of protein nutrition have left the author with the feeling that with proper application and interpretation these criteria can provide very useful information regarding nutritional status. Because these supplementary methods provide a measure of the more dynamic parameters of metabolism they would seem to offer some advantages over the conventional assay procedures e.g. anthropometry, nitrogen balance and blood protein levels. The available evidence suggests to the author that correlations of creatinine output, fasting plasma amino nitrogen levels, and metabolic rate determinations may afford a more useful and convenient

approach to the assessment of nutritional needs. Exploration of these modalities appears to be particularly worthwhile in studies involving children convalescent and aging adults because of the ease of their application.

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## CHAPTER 12

# The Amino Acid Requirements of Animals

H J ALMQUIST

*The Grange Company Modesto California*

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## I INTRODUCTION

For some time after it became possible to maintain and grow rats on purified diets the more complicated nutritive requirements of the chick could not be met except by the use of some natural feedstuffs. The chick is comparatively sensitive to the lack of a number of dietary factors in

cluding vitamin K, choline, manganese, potassium, magnesium and the more recently recognized members of the vitamin B complex. Their provision in substantially protein free forms has been a necessary prerequisite to experiments on the amino acid requirements of chicks. In recent years practically all of these factors have been identified and made conveniently available. Similarly the availability and cost of the individual amino acids have improved greatly through efforts of the large chemical suppliers. The investigator no longer must devote much time and money to manufacture a required amino acid or to provide for a certain vitamin activity.

At the inception of some of the work in which the writer was engaged, uniform conditions were maintained in respect to breed and strain of chicks used and protein or equivalent amino acid levels of the test diets. Because of the unknown but probably considerable contribution of unabsorbed yolk to the protein intake in the first week of life chicks reared on practical diets for 10 days were used. This practice also facilitated selection of chicks of uniform size vigor and growth rate leading to less individual variability in response and greater significance of data. It was observed at an early stage that responses of chicks to amino acid variations took place very fast often significantly within 24 hours unlike the slow responses obtained with many other nutritive factors. The test diets to eliminate the unknown variation in the cereal proteins contained glucose and vegetable oils to provide energy, and thus were what is now referred to as high energy diets. These circumstances were established partly by foresight and partly by fortuity.

It is the purpose of this chapter to bring up to date the information on amino acids requirements of certain animals and to mention variables affecting these requirements in association with the amino acids with which the variables became recognized.

## II YOUNG FOWLS

### A ARGININE

It had been reported that casein did not contain sufficient arginine for the rapid growth of the chick and that the ability of the chick to synthesize arginine was probably less than that of the young rat (Arnold *et al* 1936). With diets free of arginine the extreme inability of the chick to synthesize arginine was demonstrated. Rapid loss of weight was completely reversed by added arginine but not by ornithine (Klose *et al*, 1938). Later it was shown that a diet deficient in arginine could be supplemented equally well by arginine or citrulline (Klose and Almquist 1940) indicating that the chick converts the latter to arginine. The impossibility of the regeneration of arginine from ornithine by the Krebs

Henseleit cycle in chicks became obvious (Klose and Almquist, 1940 Klose *et al*, 1938 Almquist and Mecchi 1942b) This result was in harmony with the low excretion of urea and the relative absence of arginase in the chick except for small amounts in the kidney which is probably the site of the production of ornithine for the detoxication of benzoic acid (as ornithuric acid) and the simultaneous increase of urea output (Benton *et al* 1954)

It is well known that arginine is a precursor of creatine in the rat A dietary deficiency of arginine will limit creatine formation in the chick (Almquist *et al* 1941 Hegsted *et al* 1941a) The feeding of creatine creatinine guanidinopropionic acid or arginine improved both the growth rate of chicks and the tissue creatine content These results showed a "sparing action" of the first three compounds on arginine creatine being most effective (Almquist *et al* 1941 1943)

A diet based upon 35% casein glucose and soybean oil plus vitamin and mineral supplements supported improved growth of chicks when arginine or creatine was added (Wietlke *et al*, 1954 Savage and O'Dell 1956) Arginine was more effective for growth and for elevation of muscle creatine content A similar diet containing 25% casein yielded similar results (Fisher *et al* 1956) indicating that creatine can spare some but not all of the arginine requirement

In one of the early papers (Klose *et al* 1938) it was commented "Evidently part of the arginine in casein exists in such form as to be unavailable to the chick Diets based exclusively upon casein as the source of protein seem to require excessive amounts of supplementary arginine (Wietlke *et al* 1954 Fisher *et al* 1956 Krautman *et al* 1956 Snyder *et al* 1956) On the other hand when the diets contain appreciable amounts of natural feedstuffs such as alfalfa meal (Almquist and Merritt 1950) corn and soya meals (Snyder *et al* 1956) peanut meal (Young *et al* 1953) corn and soya and corn gluten meals (Krautman *et al* 1956), the arginine requirement turns out to be substantially lower and close to 6% of the dietary protein It was suggested that an unidentified factor of plant origin enhances the utilization of arginine in casein (Krautman *et al* 1956) The carbohydrate employed in the purified chick diet whether sucrose or dextrin may exert a very significant effect on the utilization of arginine (Monson *et al* 1955) It has been noted that thioracil decreases the requirement of arginine in a casein purified diet while iodinated casein increases the arginine requirement (Fluckiger and Anderson 1957)

Studies with isotopically labeled arginine comparable to work done with rats have not been reported for the chick In pigeons arginine labeled with N<sup>15</sup> in the amidine group is retained to a much greater

extent than in rats. Synthesis of arginine from labeled precursors, as in the rat, does not seem to take place in the pigeon (Block 1946). Pigeon kidney slices are comparatively inefficient in the synthesis of guanidino acetic acid from glycine and arginine (Borsook and Dubnoff 1941). Isotopically labeled arginine is employed for creatine formation distinctly less efficiently in the pigeon than in the rat (Block, 1946). While it is difficult to draw parallel comparisons between the chick and the pigeon from the work reported the impression remains that the metabolism of arginine differs appreciably in these avian species.

The growth response to an increase in supply of an essential nutrient will often be found to follow a curve of constantly diminishing increment i.e., a logarithmic curve. In such cases, plotting the data on a

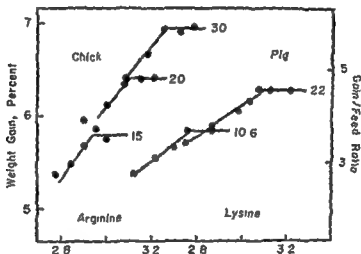


FIG. 1. The relation of daily rate of weight gain of chicks and the gain/feed ratio of young pigs to the logarithm of amino acid level in the diet at different levels of protein.

logarithmic basis will usually produce straight response lines up to the limit of response. Such limit may be set by physiological, genetic, or other nonnutritional limitations. When the limit is well defined, as by a plateau in the data, the minimal requirement then is evident from the intersection of the response and plateau lines. At a very low level of nutrient intake, the data may sometimes be seen to vary from the principle but in this case, largely because reserve stores of the nutrient in the animal or synthesis within the animal or small residual quantities in the basal diet are no longer negligible in respect to controlled intake (Almquist 1952, 1953a).

In Fig. 1 are presented several examples of the application of this principle to amino acid requirement. The left portion of this figure was constructed from data on the arginine requirements of young chickens

is affected by protein level at 15, 20 and 30% in the diet (Almquist and Merritt 1950). Noteworthy is the linear relation of rate of growth to the logarithm of the total arginine level in the diet over all protein levels. The relation reached a plateau of growth in each case as the protein level of the diet became a limiting factor. A deficiency of arginine gave the same net results as a deficiency of protein. The right portion was constructed from data on the lysine requirements of the young pig at two dietary protein levels (Brinegar *et al.* 1950).

### B HISTIDINE

Histidine has been established as an indispensable amino acid for the growth of the chick (Klose *et al.* 1938; Hegsted *et al.* 1941a). On histi-

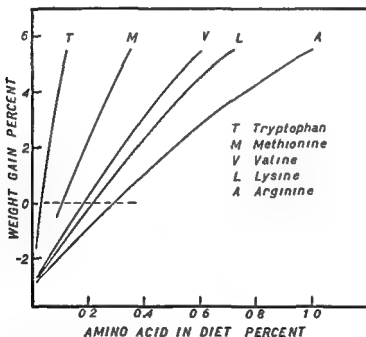


FIG. 2. The relation of the daily rate of weight gain of chicks to the percentages of certain essential amino acids in the diet.

dine deficient diets chicks did not lose weight nearly so rapidly as might have been expected. This observation may have indicated a low requirement for maintenance: small amounts of histidine in the diets or a limited synthesis (Almquist 1947) (see Fig. 2).

Solution of this problem requires a histidine-free diet which cannot be attained with available proteins. A diet based entirely upon purified amino acids in place of intact proteins was found to support good chick growth when containing approximately 0.3% histidine (Rosenberg *et al.* 1957) in agreement with previous estimations of requirement (Almquist



1956) Although a zero level was not tried the growth data at lower levels extrapolate toward zero histidine well into the zone of negative gains similarly to the cases of other indispensable amino acids for the chick (Almquist, 1947) and thus suggest that no appreciable synthesis of histidine had taken place. For a further discussion of this point see Almquist (1956 pp 140-141)

### C LYSINE

By using various combinations of proteins such as zein, edestin, and casein single deficiencies of lysine were produced in chick diets. The calculated levels of lysine as well as the levels produced by added crystalline L-lysine pointed to an adequate intake at 0.9% in the diet (Almquist, 1947; Almquist and Mecchi, 1942a). A number of later reports have confirmed this estimate of the lysine requirement of the chick and have been reviewed (Almquist, 1952). Protein level of the chick diet was found to have a direct influence on the lysine requirement (Grau, 1948) (See Table I).

TABLE I  
RELATION OF THE OPTIMAL PERCENTAGES OF AMINO ACIDS IN THE PROTEIN TO THE LEVEL OF PROTEIN IN THE DIET OF CHICKS

Percentage of protein in diet	Approximate optimal percentages for growth <sup>a</sup>		
	Lysine	Methionine plus cystine	Tryptophan
10	4.8	4.5	0.90
20	4.5	4.1	0.75
30	4.2	3.7	0.65
40	3.8	3.3	0.52

<sup>a</sup> The data were chosen as examples only and were taken from Almquist (1952) and Gruninger *et al* (1956).

The lysine requirement of the chick was given further study with diets of 20.5% protein based upon wheat gluten and sesame meal as protein sources. Lysine content was adjusted over a range of 0.70-1.40% of the diet by additions of L-lysine. The experiments, started with day-old chicks, showed a need of 1.1% lysine when the chicks grew at their best rates (Edwards *et al*, 1956). This is somewhat higher than original estimates made with mixed White Leghorn chicks that had been started on practical diet for 12-14 days. The higher requirement may be due to a relatively higher need of day-old chicks and to the fact that the chicks were a faster growing type than those originally employed. Optimal feed conversion was also obtained at about 1.1% lysine in experiments run to 6 weeks age of the birds.

Requirements have been estimated for older chicks starting with a normally raised and fed 8 week old chick and a 16% protein diet. Optimum growth and feed efficiency were observed at 0.72% total L lysine (Bird 1953). This is 4.5% of the dietary protein the same as observed with the younger chick fed a practical diet for a week or so. In utilizing the chick for the bio assay for lysine maximal weight gains were obtained at 0.91% L lysine in the diet which contained 20.8% in total, of protein and amino acids (Tsien *et al* 1957).

Diiminopimelic acid was ineffective as a lysine substitute in chick diets (Donovan 1957).

The quantitative lysine requirement of young turkeys 13% of the diet was found to be proportional to the higher protein intake which was approximately 24% of the diet as compared to 20% for the chick (Grau *et al* 1946). A summary of early reports on lysine requirement of the young poult indicated that this was about 5.5% of the dietary protein (Almquist 1952).

Using practical rations and calculated lysine contents the optimal starting protein content was found to be 28% and lysine requirement 1.3% of the poult ration. After 8 weeks the lysine requirement appeared to remain nearly constant at 4.0% of the protein (Kratzer *et al*, 1956) although the litter decreased progressively. More recent analyses indicate that the lysine content of several of the feedstuffs used is somewhat higher than indicated at the time. It is probable therefore that the above figures should be revised upward by at least 0.1% lysine in the diet.

A series of protein levels in diets for poults 0-6 weeks age yielded best gains at 27-31%. Lysine contents of the best diets were 5.7-5.9% of the protein. A lysine supplement decreased the optimal protein level to 25% the total lysine remaining at 5.9% of the protein. From 6 to 12 weeks age the optimal protein level was 23-25% and the minimal lysine requirement was at least 5.2% of the protein (Billoun and Phillips 1957).

The occurrence of a white bar decolorization in the flight feathers of young turkeys fed a lysine deficient diet depends upon the relation of the lysine level to the protein level of the diet (Kratzer *et al* 1950). D Lysine cannot replace the natural isomer for growth or feather pigmentation (Kratzer 1950). Other compounds related to lysine as metabolites or possible precursors have similarly been found incapable of replacing lysine in the diet of the poult. These compounds include proline, ornithine, homocitrulline, pipercolic acid,  $\alpha$  aminohexanoic acid and  $\epsilon$  aminohexanoic acid.  $\epsilon$  N acetyl DL lysine was utilized to some extent (Vohra and Kratzer 1957a). The role of lysine in feather pigmentation

appears to be mediated through its function in protein synthesis exclusively (Vohra and Kratzer 1957b)

A few studies have been reported on the availability of lysine in foods. *Torula* yeast was tested as a source of lysine in a simplified chick diet containing corn gluten meal and zein as chief sources of protein. At least 75% of the lysine in the yeast appeared to be available on basis of growth response, feed efficiency, and nitrogen retention, however, greater responses with yeast were obtained than could be accounted for by its lysine content. With rats, lysine of yeast was about 90% as effective as the purified L lysine (Tsien *et al.*, 1957).

The lysine in blood meal was assayed with chicks and with poult by means of a diet containing corn and sesame seed oil meal. On the basis of growth observed, lysine in blood meal was available to chicks from 64 to 85% depending upon quality of the sample. The corresponding availability of lysine to poult was 49-76% (Kratzer and Green, 1957).

Availability of amino acids in foods is extremely difficult to measure. Growth responses are often affected by some properties other than the amino acid in question. Retention of the fed amino acid in the carcass can yield only a minimum value for availability. Balance studies involving measurements of intake and excretion are complicated by the possibility of bacterial destruction of amino acids in the digestive tract. The *in vitro* effects of digestive enzymes in liberating amino acids from proteins can at best be considered only a rough approximation of what can transpire in the living animal and may suffice to show only a relatively unavailable fraction with no guarantee that the dissolved amino acid would all have become available to the animal.

Early studies on the effects of antibiotics in the diet revealed an apparently favorable effect on availability of proteins and amino acids.

The general effect of antibiotics is not to decrease protein or amino acid requirements but possibly to permit more efficient utilization (Almquist 1952). Subsequent research showing in essence that the antibiotic increases the permeability of the intestinal wall has been concisely reviewed. In addition it was shown that the absorption of  $C^{14}$  L lysine from the gut of the chick was more efficient through the thinner intestinal walls of chicks fed penicillin (Draper, 1958).

When the ability of an animal to remove amino acids from the blood by combining them into body proteins is impaired for any reason, a surplus of an amino acid may be more detrimental. For example, adding tryptophan or methionine to a diet which was already deficient in lysine further reduced the efficiency of utilization of the diet (March *et al.*, 1950).

The relation of amino acid intake and amino acid requirements to the levels of free amino acids in the blood of chicks has been reviewed (Almquist 1954 1956)

One may find in the literature reports of attempts to correct an apparent amino acid deficiency by adding at one time all the amino acids in amounts to meet known requirements. It is not surprising that these attempts have uniformly failed to restore a full balance to the diet. Universally it is true that an imbalance is not fully corrected by adding a balance to it although the over all relative severity of imbalance may be somewhat diminished. It may be stated as a general principle that an amino acid imbalance is correctable only by adding the specific components that are inadequately supplied or by adding a complementary imbalance.

#### D METHIONINE CYSTINE HOMOCYSTINE AND SULFATE

Like mammalian species the chick has the ability to grow well if given only methionine as a source of the sulfur containing amino acids and obviously can synthesize cystine under these conditions (Grau and Almquist 1943). Cystine in the diet will reduce the requisite methionine to a certain point but not appreciably below one half the level needed when cystine is not present (Almquist and Grau 1945 Grau and Almquist 1943).

The similarity in the sulfur containing amino acid metabolism of mammalian and avian species is further carried out in the utilization of homocystine. This compound which is a demethylated methionine was utilized efficiently as a substitute for methionine by the chick only when a sufficient level of choline was also present. Efficient methylation of homocystine by choline or betaine requires also the presence of vitamin B<sub>12</sub> (Jukes and Stokstad 1952). In the absence of a methylating agent homocystine may still serve as a substitute for cystine. S-Methylcystine was found ineffective either in the methylation of homocystine or the replacement of cystine (Grau and Almquist 1943 Klose and Almquist 1941).

In all probability methionine is the specific carrier of methyl groups for the conversion of guanidinoacetic acid to creatine in the chick. It has not been possible however to demonstrate more than a slight lowering of muscle creatine content by dietary deficiencies of methionine or choline or both. This is a marked contrast to the withholding of arginine and glycine. These results suggest that the methylation stage of creatine formation in the chick takes precedence over the other needs for methionine.

Work on the quantitative methionine requirement of the chick has

been reviewed in detail (Almquist, 1952). As in the example of lysine, methionine requirement was found to increase or decrease with protein level of the chick diet (Almquist, 1949; Grau and Kamei, 1950). A good deal of effort has been expended recently in relating methionine requirements to energy levels. Since the chick eats principally to satisfy caloric requirements, it will eat less of diets that are more concentrated in energy. It is obvious that for most rapid and efficient growth the high calorie diet must contain higher minimum levels of essential amino acids as well as energy, vitamins and minerals. This was demonstrated in the case of methionine, with diet energy variations from 800 to 1000 calories per pound adjusted by means of added fat (Baldini and Rosenberg, 1955). The requirements observed at the higher caloric level agree with some of the earliest estimates of methionine requirement which were determined with high energy diets. Similar further results were reported with practical diets. The improvement noted on addition of methionine was especially in reference to feed efficiency (Rosenberg *et al.*, 1955).

The principle demonstrated here is a broad and generally recognized one. It does not imply any specific relation between methionine and energy intake. Of interest in this connection is a recent report that the lysine requirement of rats increases with increase in caloric content of the diet (Rosenberg and Culik, 1955).

On the basis of recorded data on sulfur amino acids and lysine requirements of chicks, it was pointed out that the percentage of indispensable amino acid required in the protein fraction of the diet showed a consistent trend downwards as the protein level increased. Since the fraction of the protein which is metabolized for energy must increase with the level of protein fed and such utilization does not require the indispensable amino acids, it may be that lower but properly proportioned levels of indispensable amino acids will suffice in association with the fraction of protein that is utilized for formation of proteins of the growing animal (Almquist, 1952) (Table I).

It is of interest to note indications that at the higher caloric intakes the percentage of essential amino acid (methionine) required in the protein remains more constant in relation to protein level (Rosenberg and Baldini, 1957) and shows less of the drift downwards at increasing protein levels that may be noted with lower caloric intakes.

It is becoming apparent that the addition of extra calories to diets (as by fat) will tend to accentuate amino acid imbalance in the protein. These observations are no doubt related to more complete diversion of the protein for protein purposes and less for energy purposes when the diet is well supplied with calories from sources other than protein. As

the degree of utilization of protein for growth and production is enhanced essential amino acid balance assumes greater importance

Among fowls the very young are required to divert a particularly large proportion of their sulfur amino acid intake to the rapid growth of feathers which may contain as much as 10% of cystine. The rate of feather protein synthesis is compared to body protein synthesis may determine the sulfur amino acid requirement for maximal growth. It is possible that the sulfur amino acid requirement may be more intense in smaller fowls which have relatively more surface area per unit of body weight (Almquist 1952) (see also chapter by H. H. Mitchell). The presently available data on sulfur amino acid requirements indicate the relative needs of young fowls as seen in Table II.

TABLE II  
COMPARISON OF RELATIVE REQUIREMENTS OF SULFUR AMINO ACIDS IN THE DIET OF YOUNG FOWLS

Fowl	Approximate requirement as per cent of the protein in the diet at optimal protein levels		
	Methionine	Cystine	Total
White Leghorns	25	20	45
Heavy meat chickens	23	17	40
Turkeys	20	13	33

This comparison of relative requirement of sulfur amino acids in the protein fraction of the diet indicates that smaller fowls need higher proportions of sulfur amino acids in the dietary protein. On the other hand some evidence has been reported which suggests a genetic difference among strains of chickens in the ability to utilize methionine (McDonald 1957).

The presence of an antienzyme in raw legumes such as the soybean is well known. The apparent interference with methionine availability in the example of raw soybean meal has long been recognized. It was pointed out that this apparent interference was probably merely coincidental with the fact that soybean protein does not contain enough methionine to meet the needs of fast growing animals (Almquist and Merritt 1951).

Subsequently it has been shown by proper choice of proteins fed in conjunction with an effective level of raw soybean enzyme inhibitor that a partial deficiency of lysine or arginine or isoleucine or tryptophan may also be made more severe by the presence of the antienzyme from the raw soybean (Almquist and Merritt 1951, 1952). It is evident therefore that the methionine case is only part of a more general phenomenon which may be demonstrated with other amino acids. There is no evi-

dence nor reason to assume, that the methionine in raw soybean meal is unavailable

Synthetic DL methionine appeared to be essentially completely utilized as a supplement to natural feed diets moderately deficient in the amino acid (Grau and Almquist 1943) The hydroxy analog, DL  $\alpha$  hydroxy  $\gamma$  methylmercaptobutyric acid, and the corresponding amide were well utilized by the chick for growth (Bird, 1952) It has been reported that the racemic hydroxy analog was slightly more effective than racemic methionine but equal to L methionine in satisfying the requirement of the chick, while D methionine was less effective than any of the other compounds (Gordon and Sizer 1955a) This was taken to indicate that the hydroxy analog was capable of a more complete conversion to natural methionine

DL Methionine was more effective than its hydroxy analog for promoting growth of chicks fed a simplified diet which was based on isolated soybean protein to provide a total of 11-13% protein This low protein diet did not provide sufficient extra nitrogen to permit efficient conversion of the analog to methionine in which process an amino group must replace the hydroxy group Urea and ammonium citrate improved the growth response to the analog apparently by providing the required extra amino groups (Sullivan and Bird 1957) A subsequent report on experiments conducted similarly has indicated no difference in the relative efficiency of utilization of DL methionine and its hydroxy analog (Machlin and Gordon 1957)

Early studies on methionine requirement of the turkey have been reviewed (Almquist 1952) Results to 12 weeks age with turkey diets of 24 26 and 28% protein and methionine supplements of 0.05 and 0.10% have shown that growth increased directly with protein level When caloric content of diets was increased 100 calories per pound by fat additions, growth was depressed however there was a marked growth response to 0.05% added methionine and little further increase to the higher level Feed conversion improved progressively with both supplementary additions (Ferguson *et al*, 1957) It may be calculated that at least 0.55% total methionine was needed at the 28% protein level in agreement with former requirement estimates This work furnishes a striking illustration in support of the prior observations concerning the influence of caloric intake on amino acid balance

Poults kept on diets of 28% protein to 8 weeks showed best growth at a level of 0.51% methionine For the period 9-16 weeks the protein level was 22% and best methionine level 0.41% From 16 to 24 weeks the protein level was 17% and best methionine level about 0.34% (Donovan *et al* 1955) At all protein levels the optimal proportion of meth

ionine in the protein tended to remain the same (The above estimates are based upon calculations by the reviewer)

At a constant level of protein, 26% of the diet, the methionine requirement of the poult varied directly with the energy content of the diet (Baldini *et al*, 1957). A logarithmic analysis of the data (Almquist, 1952-1953a) indicates that at the higher caloric intakes the methionine requirement for best rates of gain and feed efficiency became constant at about 0.57% of the diet. The cystine content of the diet was not raised and it seems possible that some of the supplementary methionine could have been replaced as well by supplementary cystine.

Reports on the utilization of labeled S in sulfate by chicks have a bearing on the sulfur amino acid requirement. While such sulfate is only to a negligible extent incorporated into amino acids it does appear in certain body constituents as taurine and in the chondroitin sulfate of cartilage (Machlin and Pearson 1956; Machlin *et al* 1955a; Gordon and Sizer 1955b). It therefore meets part of the total sulfur requirement of the chick, a part which would have to be secured from the sulfur amino acids if from no other source. Data showing growth promoting effects of sulfate added to a low sulfate low methionine diet for chicks have been presented (Machlin and Pearson 1956; Machlin *et al* 1955a). It appears that demonstration of the effect requires a diet low in total sulphur and in sulfate.

After injection of cystine containing  $S^{35}$  into the incubating egg the labeled S was recovered from the hatched chick. An average value of 10% was present in taurine, 12% as sulfate, 13% as methionine and 49% as cystine. When S labeled methionine was injected 91% was recovered as taurine, 10% as sulfate, 43% as cystine and 35% as methionine (Machlin *et al* 1955b).

It is quite possible that sulfates are present in some crude sources of "unknown growth factors". Fortunately many of the simplified diets used to estimate amino acid requirements were well supplied with sulfates used as convenient forms to provide certain required metallic elements such as magnesium, manganese, etc.

### E. TRYPTOPHAN

The essential nature of tryptophan in the diet of the chick has been well demonstrated (Almquist and Mecchi, 1941; Grau and Almquist 1944a; Klose *et al* 1938). In contrast to the rat and mouse the chick did not appear to utilize the unnatural form and racemic tryptophan was therefore only half as active for the chick as the natural form (Grau and Almquist 1944a). With other types of diets and chicks evidence has been obtained that the chick does utilize the D isomer to a



variable degree, possibly with the assistance of microorganisms. These studies have been reviewed (Almquist, 1952).

In further studies of tryptophan requirement chicks were fed diets containing acid hydrolyzed casein in which tryptophan has been largely destroyed. The diet was supplemented with adequate amounts of arginine, glycine, methionine, and niacin. The testing involved growth studies starting with 10 day old normal male chicks fed diets of 10, 20, 30, and 40% protein. Various levels of L tryptophan were added. The authors concluded that the total tryptophan for maximal gain was 0.09, 0.14, 0.18, and 0.20% at the respective protein levels (Griminger *et al.* 1956). This agrees with findings with other essential amino acids that the requirement increases with the protein level, but usually at a slower rate (Almquist, 1952) (Table I).

It is evident that the birds did not grow well—in fact retarded growth with hydrolyzed protein and amino acids diets has been known for years (Luckey *et al.*, 1947, Stokstad 1941), and is possibly due to physiological causes not to any deficiencies. Among such causes may be the increased caloric requirement noted with amino acid diets by Rose and co workers (1954), and appetite factors (Almquist 1947). There is some question whether requirements measured at anything below good growth rates will represent the full requirement of the animal.

The reviewer has analyzed the data from the standpoint of feed efficiency versus log tryptophan content of diet. Feed efficiency is sometimes more consistent than growth rate when diets permit only subnormal growth. The data fall on a plot which shows as have former examples with arginine and lysine (Almquist 1953b) that feed efficiency is a continuous function of the log tryptophan intake over all protein levels, with plateaus in feed efficiency attained as the gross protein level of the diet becomes the limiting factor in each case. While the data are not satisfactory for assessment of practical tryptophan requirement they do fit remarkably well into the concepts already illustrated in the cases of other amino acids.

By means of a diet containing 0.036% tryptophan it was shown that D tryptophan is very poorly utilized by the oral route but much more efficiently utilized when given to the chick by subcutaneous injection. This work suggests that inefficient absorption is a primary cause of poor utilization of dietary D tryptophan (Morrison *et al.* 1946).

Tryptophan which is a precursor for niacin synthesis in mammals is also effective in counteracting the pellagic syndrome induced in niacin deficient chicks (Briggs 1945, Briggs *et al.*, 1946).

In agreement with an earlier report (Grau and Almquist 1944a) L tryptophan was found twice as active as the DL form in the chick for

growth and also for niacin replacement when the carbohydrate in the diet was glucose. Replacement of glucose by starch permitted significant utilization of the D isomer apparently through the aid of microorganisms (Anderson *et al* 1950). The replacement of sucrose by dextrin in a purified chick diet was also found to enhance the efficiency of a tryptophan supplement (Benton *et al* 1951).

The relation of niacin to tryptophan in the chick was further investigated with a diet of corn isolated soybean protein gelatin methionine, plus vitamins and minerals. The tryptophan added was the DL form which introduces an uncertainty involving the magnitude of utilization of the D isomer. The basal diet apparently contained 0.15% tryptophan (calculated 0.19%). At this level approximately 13–15 mg of niacin per pound of diet were required for optimal growth. When 0.1% DL tryptophan was added the niacin requirement decreased to 8–9 mg (Patterson *et al* 1956).

Studies on the tryptophan requirement of the poult have been reviewed (Almquist 1952).

### F PHENYLALANINE AND TYROSINE

Chicks lose weight rapidly on diets which do not contain phenylalanine (Almquist and Grau 1944 Hegsted 1944). Phenylalanine may meet all the requirement for these aromatic amino acids but tyrosine has a growth promoting effect only when the phenylalanine level is not optimal (Almquist and Grau 1944 Grau 1947). The relation between these amino acids in the chick is similar to that already known in other species.

A first estimate of the phenylalanine requirement of the chick placed it at not more than 0.9% of the diet (Almquist 1947). A later report placed this requirement at 0.6–0.8% of the diet but the growth rates of the chicks were much below optimal (Grau 1947).

Further studies on the phenylalanine requirement of the chick have been reported (Fisher 1956). The first experiment was conducted with a diet of approximately 20% protein provided from wheat germ meal gelatin and amino acids so as to contain 0.5% L phenylalanine. Weight gains were about half those to be expected from the type of chick used. However the average data show a response of weight gains to additions of L phenylalanine up to a total dietary content of 0.9%. A plot of the feed efficiency data against log L phenylalanine shows a close linear relation up to 0.9%. In a second similar experiment best results were again obtained with 0.9% L phenylalanine.

The phenylalanine requirement was further studied using a diet containing about 18% of amino acids including 0.7% L tyrosine in place of



limited to increase the total isoleucine content to approximately 0.8% of a 27% protein diet. Isoleucine appeared to be inactive (Kratzer *et al.* 1952).

### I. VALINE

Valine requirement of the chick was proven with amino acids diets (Almquist and Grau 1944, Grau and Peterson 1946, Hegsted 1944). As with other essential amino acids the lack of valine in the diet caused an immediate loss of weight and early death. Valine requirement was estimated as 0.7% (Grau and Peterson 1946) and 0.8% of the diet (Almquist 1947).

### J. THREONINE

An amino acids diet was used to demonstrate threonine requirement of the chick (Almquist and Grau 1944). Optimal threonine content was estimated as approximately 0.6% of a 20% protein diet for chicks (Almquist 1947). A few other reports having a bearing on the threonine requirement have been reviewed (Almquist 1952) without change in the estimate of requirement.

### K. GLYCINE

A dietary supply of glycine is necessary for optimal growth of the young chick (Almquist and Grau 1944, Almquist and Mecchi 1940, Almquist *et al.* 1940, Hegsted *et al.* 1941b). A deficiency of this simplest of amino acids in the chick results in poor growth, reduced creatine formation, a generalized weakness and muscular attenuation (Almquist and Mecchi 1940, Almquist *et al.* 1941) and poor feather formation (Hegsted *et al.* 1941b, Jules 1941). The requirement for glycine and arginine appears more acute in a more rapidly feathering breed of chickens since feather protein contains large percentages of these amino acids (Hegsted *et al.* 1941a). The chick has a limited ability to conduct synthesis of glycine (Almquist and Grau 1944, Almquist and Mecchi 1942b, Hegsted 1944). This is unaffected by the omission or inclusion of serine (Almquist and Grau 1944), glycolic acid, betaine,  $\beta$ -alanine or choline, but is apparently favored by the addition of acetates to the diet (Almquist and Mecchi 1940). The glycine requirement of the young chick was found to be approximately 1.5% of the diet when provided as free glycine or 1.0% when provided in combined form (Almquist and Mecchi 1942b).

A reinvestigation of the glycine requirement of young chicks confirmed the estimated requirement of 1.5% of free glycine in a 20% protein diet. The growth effect of the glycine was apparently not due to nitrogen alone, as it was not replaceable by ammonium compounds.

(Wixom *et al* 1955, 1958) The basal diet contained approximately 1.2% serine

Serine glycolic acid and betaine did not replace glycine (Wixom *et al*, 1958), in agreement with an earlier report (Almquist and Mecchi 1940) Aminoethanol—but not mono and dimethyl aminoethanol (Wixom *et al* 1958)—or choline (Almquist and Mecchi 1940) could replace glycine In similarity to betaine di and monomethyl glycine did not replace glycine (Wixom *et al* 1958) It was suggested that conversion of aminoethanol to glycine might proceed via glycolaldehyde glycolic acid glyoxylic acid however there is nothing to rule out a possible direct oxidation of aminoethanol to glycine without intermediate deamination Several studies with free glycine supplements have indicated requirements of 1.5% or higher while on the other hand several practical protein concentrates used to provide all the protein in the diet, are not improved by a glycine supplement although furnishing no more than approximately 1% of glycine, on the basis of present analyses Until this situation is further clarified it seems proper to adhere to the 1% minimum glycine level for practical diets

At levels higher than 2% of the chick diet, glycine appears to be harmful to growth (Almquist *et al*, 1940) Large doses are toxic to hens (Patton 1939) This toxicity in chickens may be related to the nicotinic acid (niacin) content of the diet, glycine or gelatin in a niacin deficient diet will accelerate the development of symptoms of chick pellagra (Briggs 1945 Groschke and Briggs 1946) but the addition of sufficient niacin will enable the chick to tolerate as much as 6% of glycine in the diet Niacin is evidently concerned in the metabolism of glycine as guanine and alanine in the chick It may be significant that the formation of cartilaginous tissues which are rich in these amino acids is deranged in the niacin deficient chick (as manifested by perosis)

It has been suggested that glycine requirement and glycine toxicity may be modified by the age of the chick In older chicks apparently, glycine and creatine syntheses are well established hence dietary glycine is more likely to constitute a toxic surplus (Albert *et al* 1956) In addition to niacin both folacin and vitamin B<sub>12</sub> can alleviate glycine toxicity (Marchlin *et al* 1956) No clues could be found in tissue and blood levels of glycine or of folacin or in the employment of glycine metabolites as to the mechanism of the effect of folacin (Naber *et al* 1956)

Young poults fed a ration based upon casein arginine and cystine showed growth response to additions of glycine to build the total to 0.90% of the diet (Kratzer and Williams 1948) The status of glycine toxicity was reviewed and further work with the turkey presented to show that in low folacin diets the addition of glycine caused depressed

growth increased mortality and cervical paralysis all preventable by folacin (Kratzer and Lantz 1957)

### L. OTHER AMINO ACIDS

Proline, hydroxyproline and glutamic acid have been given no further study since an early report on temporary or slight growth retardation following their omission from chick diets. No such influence could be attributed to lack of alanine or aspartic acid or serine. The chick appeared to require glutamic acid for best growth (Almquist and Grau 1944) (Table III)

TABLE III  
THE ESSENTIALITY OF AMINO ACIDS FOR CHICKS

May be absent without detriment to chick	Required under certain conditions	Required for normal growth	
Alanine	Cystine	Arginine	Lysine
Aspartic acid	Tyrosine	Glutamic acid	Methionine
Hydroxyproline		Glycine	Phenylalanine
Proline		Histidine	Threonine
Serine		Isoleucine	Tryptophan
		Leucine	Valine

Certain aspects of carcass analyses for amino acids and of free amino acids in the blood which have a bearing on amino acid requirements, have been reviewed (Almquist 1956)

### III ADULT FOWLS

Information available on the requirements for amino acids by adult fowls is mostly of a quantitative nature. The early phases of this work have been reviewed (Almquist 1952)

#### A METHIONINE

Supplemental methionine in a corn soybean meal diet for laying chickens was carefully tested for effects on egg production egg weight shell thickness feed required per dozen eggs hatchability and bird weight. There were no appreciable effects attributable to the methionine. The methionine contents of the diets determined by analysis agreed very closely with contents that may be calculated from modern tables and were on an average 0.28% methionine in a 15% protein diet (Heywang 1956). This is the same as the estimate of the optimal methionine content based upon reports previously covered by the reviewer (Almquist 1952)

Reduction of cannibalism in laying hens followed addition of synthetic methionine to a laying mash. The total content of methionine required to prevent these vices appeared to be 0.30% of the diet (Neal 1956).

A diet of peas, glucose, gelatin, etc. was employed with laying hens (Welch and Couch, 1955). The methionine assay on the peas indicated 0.42% which is much higher than has been reported elsewhere. The total diet was calculated to contain 0.26% methionine; this actually may have been less. Production was poor with the basal diet. Homocystine or vitamin B<sub>12</sub> alone did not affect production, but the combination improved production. Choline addition with homocystine was much more effective, but not equal to methionine. The best production was observed in groups supplemented with methionine and especially with methionine, choline and vitamin B<sub>12</sub>.

#### B LEUCINE

Laying rations high in wheat, but containing commonly used amounts of fish meal, meat scrap, soybean and alfalfa meals, dried whey, and other supplements may be deficient in leucine. This was shown by small but practical improvements in egg production, feed efficiency and body weight when corn gluten meal (rich in leucine) was substituted for soybean meal or when 0.3% L-leucine (technical grade) was added to the ration. It would appear that a diet of 17.5% protein with 1.26% leucine and one of 15.3% protein with 1.14% leucine, were both mildly deficient in this amino acid (Anderson and Draper, 1956). It is not known whether all of the 0.3% L-leucine addition was required.

#### C THREONINE

A preliminary report has indicated that the laying hen needs L-threonine at a level of 0.42% of the diet for good egg production and maintenance of body weight (Adkins *et al.* 1957).

#### D OTHER AMINO ACIDS

The formulation of a successful amino acids diet for laying hens and a more complete classification of essential amino acids for laying hens have been reported (Fisher and Johnson 1956; Johnson and Fisher, 1956). Good production was maintained and in a limited experiment, good fertility and hatchability observed. The essential amino acids for egg production were found to be the same as those of the growing chick, with the exception of glycine. Glutamic acid was semi-essential, i.e. required for best production. It has been found to be required for best chick growth (Almquist and Grau 1944). Deletion of any other essential

amino acid caused immediate cessation of production. The hens appeared to have only very small reserve protein which was quickly exhausted on the deficient diets.

Barred Rock pullets in laying cages were fed a basal synthetic diet adequate in all nutrients except arginine and glycine. Most efficient production was obtained only when both arginine and glycine were restored to adequate levels in the diet. The body weight gain was influenced to a certain degree by glycine but not by arginine or a combination of the two. This preliminary report indicates a joint need for glycine and arginine in the diet of the laying hen (Menge *et al.*, 1956).

In Table IV recent data on amino acids in poultry products and dietary requirements have been compared. With the exception of lysine the egg is markedly richer in the indispensable amino acids as com-

TABLE IV  
COMPARATIVE AMINO ACID COMPOSITION OF POULTRY PRODUCTS<sup>a</sup>

	Egg edible parts <sup>b</sup>	Chicken muscle <sup>b</sup>	Egg edible parts	Hen dietary <sup>b</sup> requirement
Arginine	6.7	6.7	4.2	+ <sup>d</sup>
Histidine	2.4	2.0	1.5	
Lysine	6.1	8.4	3.8	3.3
Methionine	3.3	2.4	2.1	1.9
Cystine	2.3	1.0	1.4	
Phenylalanine	5.8	4.1	3.6	
Tyrosine	4.1	2.7	2.6	
Tryptophan	1.6	1.0	1.0	1.0
Threonine	5.0	4.0	3.1	3.0
Leucine	9.2	8.2	5.7	8.0
Isoleucine	6.0	4.2	3.7	3.3
Valine	7.4	4.1	4.6	
Glycine	3.6	5.7	2.3	+ <sup>d</sup>

<sup>a</sup> For complete tables of amino acid analyses see Block and Weiss (1956).

<sup>b</sup> As percentage of the crude protein (% N  $\times$  6.25).

<sup>c</sup> Per unit of tryptophan.

<sup>d</sup> Indicates a positive requirement; minimum level not yet determined.

pared to chicken muscle. When the egg composition is recalculated on basis of tryptophan content as unity, the same as in muscle, it is evident that the agreement in proportions of most indispensable amino acids is quite close, with the exceptions that the egg proteins are relatively lower in arginine and lysine. This may indicate that the hen is better adapted to utilize proteins in cereals and many seeds which are deficient in lysine for the growing bird.

Comparison of the third column with the fourth indicates that the



relative proportions of indispensable amino acids in the egg agree approximately with those required in the hen diet with the exception of leucine. Valine requirement of the hen could be expected to be approximately 50% and phenylalanine requirement approximately 35% of the dietary protein.

#### IV YOUNG SWINE

##### A METHIONINE AND CYSTINE

A 21% protein diet, based on oxidized casein and gelatin as the sole source of protein and 0.3% DL tryptophan, was found by microbiological analysis to contain 0.1% methionine and 0.01% cystine. Poor gains were but slightly improved when cystine alone was added (Shelton *et al.*, 1951b). In the case of diets containing 0.31% cystine or more, the reviewer has plotted the observed gain:feed ratios against the logarithms of the methionine levels of the diet. This treatment indicates an optimal methionine level of 0.38%.

Data from another study (Curtin *et al.*, 1952c) have been similarly analyzed. Two experiments were reported, one of which is in close agreement with that discussed above (Shelton *et al.*, 1951b). Both experiments indicate an optimal methionine content of 0.41% in the presence of an ample level of cystine (0.38%) for a 22% soybean protein diet.

Another report (Curtin *et al.*, 1952b) of results, with a diet containing isolated soybean protein and yeast as the sources of protein, was subjected to similar analysis. This indicated a methionine requirement of 0.45% in a ration containing 0.26% cystine and 22% protein. It is probable that the cystine level could have been raised to 0.30% to good effect, and the methionine correspondingly lowered to 0.41%.

The methionine and cystine need of the young pig was further studied with diets which were based upon isolated soybean protein, starch, dextrose, and corn oil, plus mineral and vitamin supplements. Analysis indicated methionine 0.15, cystine 0.17, and lysine 0.71% in a 12.6% protein diet. Approximately 0.10% added DL methionine was sufficient for maximal rate of gain. However, the next level added 0.20% of DL methionine or of methionine hydroxy analog yielded best feed efficiency (Becker *et al.*, 1955a). The reported cystine content seems unusually high in view of the fact that the cystine content of this protein preparation as reported by the manufacturer is much less than the methionine content.

##### B LYSINE

A 10.6% protein diet in which the nitrogen was furnished by linseed meal with supplements of methionine and histidine, was varied by the addition of L lysine. Maximal rate of gain and feed efficiency were at

tained at 0.58% total lysine. A 22% protein diet based on sesame meal required fortification to 1.17% lysine for the attainment of maximal rate of gain and best feed efficiency. A third diet based on meat and bone scraps, zein and wheat gave maximal results at more than 1.00% but not more than 1.20% total lysine (Brinegar *et al.*, 1950b). In this diet the reported lysine content is approximately 0.2% higher than would be expected from published analyses of similar ingredients. If corrected on this basis the data from the third experiment are in close agreement with those of the second. All three experiments on a plot of gain/food ratio against log total lysine form one continuous line with plateaus developed as the limiting effect of each protein level is attained (Fig. 1). The lysine content of the optimal diet appears to be approximately 1.2 or 5.5% of the protein. This report is in agreement with findings with other species in that the essential amino acid requirement is closely related to total protein intake.

A ration containing 23.8% of protein furnished as zein, gelatin and amino acid supplements was fed to young pigs (Shelton *et al.*, 1951a). Lysine was varied from the assayed basal diet content of 0.50% up to 1.40%. Maximal gains and best feed efficiency were apparently attained at 1.0% total lysine; however the data beyond this point are not sufficiently consistent to define a plateau of gain.

Lysine requirement of the suckling pig was studied using a ration with dry skim milk and sesame oil meal as sources of protein. Lysine in the ration was assayed to be 0.53% and protein 14.3%. DL Lysine was added. Maximal gain and feed efficiency were attained with a total of 0.93% L lysine (assuming the D isomer inactive). This is equivalent to 6.6% of the protein and may indicate that the requirement of the very young pig is higher than that of the weaned pig (Hutchinson *et al.* 1957b). A further study with the weaned pig fed a corn and sesame oil meal diet indicated that the minimum requirement was not over 0.52% L lysine in an 11.7% protein diet equivalent to 4.5% lysine in the protein (Hutchinson *et al.* 1957a). The pigs were fed twice daily rather than *ad lib* and gains were not as good as normally expected.

### C HISTIDINE

The essential nature of histidine for the young pig was further studied with a simplified diet of mixed amino acids. Pigs receiving no histidine supplement grew poorly or not at all. When histidine was added to their diets the pigs showed improved appetites and fair rates of growth. Evidently histidine is needed for normal growth of young pigs (Eggert *et al.* 1955).

Histidine requirement of baby pigs has been further studied by use

of dried whey and amino acid mixtures. At an equivalent of 20% protein, weight gains improved when L-histidine was raised up to 0.30% of the diet while a 16% protein diet showed improvements up to 0.20% L-histidine. Definite depressions were noted at higher histidine levels. The optimal levels are equivalent to 1.2–1.5% histidine in the protein. It may be added that in both experiments the gains were a linear function of the log of the histidine in the diet, up to the levels cited (Rechcigl *et al.*, 1956).

#### D TRYPTOPHAN

A zein and gelatin combination plus amino acids was used as a protein source for the study of tryptophan requirement. Microbiological analysis of the diet indicated less than 0.1% tryptophan. DL-Tryptophan was added by 0.1% stages to 0.4%. As good a growth was attained by 0.2% added DL-tryptophan as at any higher level while 0.1% was clearly inadequate (Shelton *et al.*, 1951c). Since it has been shown that other species can utilize D-tryptophan to some extent, it is probable that the pig can do so. Nitrogen balance studies with young pigs have indicated that some utilization of D-tryptophan does take place (Thompson *et al.*, 1952).

It was concluded that no more than 0.115% tryptophan was needed in a pig diet containing 15.3% protein, provided principally by corn and fishmeal (Becker *et al.*, 1955b). The data are too variable to justify an exact estimate of tryptophan requirement. In particular results with the 0.115% tryptophan level are so out of line with adjacent results as to appear unreliable. The data more consistently indicate that the requirement is higher than 0.115%. The value of tryptophan assigned to the basal diets was 0.075–0.078% which is surprisingly low in view of the ingredients used. Calculation from modern tables of amino acid composition indicate that the tryptophan content may have been as much as 0.18% of the diet.

Further studies on the tryptophan requirement of the baby pig have been reported. Unfortunately this work employed a hydrolyzed casein diet and DL-tryptophan. In the absence of niacin more tryptophan was required. Since the utilization of the D-isomer is undoubtedly affected by many factors including the level fed, it is difficult to interpret the data in terms of natural tryptophan requirement (Firth and Johnson, 1956).

#### E ISOLEUCINE

Requirement of isoleucine was estimated by means of a blood flour diet with amino acid supplements. Blood flour is relatively deficient in isoleucine. The diets contained 20.8–22.1% crude protein and the iso

leucine was supplied in the form of DL isoleucine. Since no animals are known to utilize D isoleucine it was assumed that only the L component was active. Maximal gain and feed efficiency were attained at more than 0.58% isoleucine but not more than 0.70% (Brinegar *et al.* 1950a). The isoleucine content found by analysis of the diet compares closely with that obtained by calculation from existing data. A log plot of these data indicates a maximum requirement not much more than 0.63% of the diet or approximately 2.8% of the protein.

A further study of the isoleucine requirement of the weanling pig was made with the two diets of 13.4 and 26.7% protein based on soluble blood flour and supplements of L isoleucine. Minimum isoleucine levels for optimal results were 0.46% of the 13.4% protein diet and 0.65%, or slightly more of the high protein diet. As a percentage of the protein less isoleucine was required at the higher protein level. An isoleucine deficiency appears very unlikely in practical diets (Becker *et al.* 1957).

#### F LEUCINE

A preliminary mention of results on leucine requirement studies with the very young pig (Sewell *et al.* 1953) indicates a requirement between 4 and 5% of the dietary protein.

#### G THREONINE

Requirement for threonine was demonstrated with diets containing an amino acid mixture in place of protein. Pigs grew on the diet containing 10 essential amino acids but lost weight when threonine was not included. With a corn and amino acids diet containing 13.2% crude protein it was shown that the threonine requirement was not more than 0.4% of the diet (Shelton *et al.* 1950).

Studies on the requirement of the very young pig for threonine were conducted with a simulated milk diet containing isolated soybean protein and amino acids (Sewell *et al.* 1953). When placed on a log plot the data show a linear relation between gain:feed ratio and log total L threonine in the submaximal zone. The maximum is not well defined. The requirement is probably between 0.73 and 0.92% of the diet which contained approximately 25% protein.

#### H VALINE

A 12.8% protein diet based upon corn and amino acids as sources of protein was supplemented with DL valine. The daily gain of pigs reached a well defined plateau at 0.4% L valine (Mertz *et al.* 1953). Since there was only one clearly submaximal datum the response line cannot be ac-

curately placed and the requirement may, possibly, be less than 0.4% in diets of this protein content

### I PHENYLALANINE

Data on this amino acid are not yet sufficient for more than a rough estimate that the requirement is not more than 0.5% of a 20% protein diet (Mertz *et al.* 1954)

### J OTHER AMINO ACIDS

A diet containing only the 10 indispensable amino acids and diammonium citrate as sources of nitrogen was fed to young pigs. Pigs receiving all 10 indispensable amino acids showed good average daily gains of 1.12–1.29 lb. Pigs receiving no arginine showed much poorer gains. Those receiving no leucine or valine lost weight. Animals receiving no added phenylalanine barely maintained weights; however, their diets contained traces of phenylalanine. In addition to other amino acids already mentioned, arginine and phenylalanine may be added to the list required by the young pig for good growth (Mertz *et al.*, 1952).

After analyses of pig tissues for amino acids, and using lysine as the common standard of comparison, calculations were made of the amino acid dietary requirements (Curtin *et al.*, 1952a). The agreement of estimates from tissue analyses and actual growth requirements is fairly good in the cases of isoleucine, leucine, threonine, and valine, but quite divergent in the cases of methionine and tryptophan.

"Essential Amino Acid Index" was calculated from the amino acid composition of practical protein sources and related to biological value of the proteins as determined with pigs. A correlation of 0.77 was found (Armstrong and Mitchell 1955). The essential amino acid index amounts to an average of the degrees of adequacy of the supply of each essential amino acid. By such calculation an amino acid, only moderately deficient has an effect on the index despite the fact that another amino acid may be more acutely deficient. It remains to be seen whether a protein source of low biological value because of one serious essential amino acid deficiency, is affected at all in respect to this biological value if another essential amino acid is varied from adequacy to moderate deficiency.

A diet of corn, soybean meal, trinkage, and wheat shorts as protein sources was fed to weanling pigs. Additions of lysine, tryptophan, and methionine improved growth rate and feed efficiency. The requirements of the weanling pig appeared to have been met by 5.0 lysine, 3.5 methionine plus cystine, and 1.0 tryptophan, expressed as a percent of the

protein. Protein levels were 14, 16, and 18% (Pfander and Tribble 1955).

Practical diets containing 0.13% tryptophan, 0.23% methionine, and 0.63% lysine in a total of 14–16% protein were adequate for the pig from 40–100 lb (Becker *et al.* 1951). The reported tryptophan contents of the diets appear to be much lower than calculations would indicate.

The addition of either tryptophan, methionine, or lysine to a 15.9% protein diet for 70 lb pigs did not improve the nitrogen retention, presumably because this level of protein is in excess of needs of pigs of the size used (Meade 1956a).

The nitrogen balance of the young pig fed diets of approximately 12, 14, and 16% protein was studied with methionine, lysine, and tryptophan variables. In general, best nitrogen retention and weight gains were observed at 16% protein. At this level of protein, results were not significantly improved by raising the methionine level above 1.7% of the protein, although an upward trend may be noted. Tryptophan at about 1% of the protein appeared to be adequate. Lysine was adequate at the minimum level fed, 4.6% of the protein (Meade 1956b; Meade and Teter 1956).

## V SHEEP

The sulfur requirement of lambs was estimated with a simplified diet of starch, glucose, straw, wood pulp, vegetable oils, minerals, and vitamins together with 4% urea to provide 92% of the total nitrogen. Sulfur was provided as the element as sodium sulfate and as methionine. An optimal requirement for each form was found with the growth efficiency increasing in the order sulfur, sulfate, methionine. Only the latter improved wool yield. The total requirement for methionine was 0.64% of the diet (Albert *et al.* 1956). In practical diets, cystine would be expected to equal to replace in part or perhaps to exceed the efficiency of the methionine.

## VI FISHES

The formulation of a successful amino acids diet for salmonoid fishes has been described (Halver 1957). Small Chinook salmon were found to require for good growth the same 10 amino acids required by the young rat and the young pig (DeLong *et al.* 1956). However, the fish showed no ability to synthesize arginine, so it may be said that they resemble the chick qualitatively in amino acid requirements.

## VII SUMMARY

Comparative amino acid requirements of various species, as indicated in the reports reviewed, have been summarized in Table V.

TABLE V  
REQUIREMENTS FOR INDISPENSABLE AMINO ACIDS<sup>a</sup>

Amino acid	Young chick (per cent of diet)	Young turkey (per cent of diet)	Laying hen (per cent of diet)	Young pig (per cent of diet)	Young rat (per cent of diet)	Adult human (grams per day)
Arginine	1.2	1.6 <sup>b</sup>	+ <sup>c</sup>	0.3	0.2	0.0
Histidine	0.3			0.3	0.4	0.0
Lysine	1.0	1.5	0.50	1.1 <sup>b</sup>	1.0	0.80
Tryptophan	0.20	0.26 <sup>b</sup>	0.15	0.2	0.2	0.25
Methionine	0.45	0.53	0.28	0.37 <sup>b</sup>	0.4	0.22
Phenylalanine	0.8			0.70 <sup>b</sup>	0.7	1.10 <sup>d</sup>
Leucine	1.4		1.2	1.2	0.8	1.10
Isoleucine	0.6	0.8 <sup>b</sup>	0.5	0.6 <sup>b</sup>	0.5	0.70
Valine	0.8			0.6 <sup>b</sup>	0.7	0.80
Threonine	0.6		0.45	0.6 <sup>b</sup>	0.5	0.50
Glycine	1.0	1.0 <sup>b</sup>	+ <sup>c</sup>		0.0	0.0
Approximate protein level	20	28	15	20	20	

<sup>a</sup> When the diet contains all other amino acids of significance for requirements plus nonessential amino acids available nitrogen

<sup>b</sup> Estimated from requirement found with protein level different from that indicated

<sup>c</sup> Indicates positive requirement minimum level not yet determined

<sup>d</sup> Includes quota for synthesis of tyrosine

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## CHAPTER 13

# Amino Acid Supplementation of Foods and Feeds

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## I INTRODUCTION

Amino acid supplementation of foods and feeds brings about many beneficial results. Of practical importance is the attainment of a better balanced protein and thereby of a better balanced diet, an extension or saving of the available protein supply, and an improvement in the efficiency of protein and food utilization. In general, in order to be successful, amino acid supplementation of foods and feeds must not only be effective but also economical.

Amino acid supplementation of foods and feeds has been practiced for a number of years. All the important amino acids are commercially available. The two most critical ones, methionine and lysine, are manu-

factured by total synthesis: DL-Methionine and its  $\alpha$ -hydroxy analog which can replace methionine in the presence of an amino nitrogen donor are low cost ingredients for food and feed supplementation. Lysine marketed as the monohydrochloride is no longer the expensive chemical which it was only a few years ago. When the demand for lysine will increase to the point of large scale production further price reductions can be visualized.

The world wide shortage of protein and the success enjoyed in overcoming specific protein deficiencies by supplementation with essential amino acids has created the need for a broader understanding of the scientific rationale of amino acid supplementation of foods and feeds and of the benefits which may be derived therefrom. It is the purpose of this chapter to discuss the principles governing successful utilization of amino acids in foods and feeds and to comment on some of the methods available to demonstrate the nutritional improvement which can be attained by amino acid supplementation.

## II GENERAL PRINCIPLE OF AMINO ACID SUPPLEMENTATION OF FOODS AND FEEDS

### A THE CONCEPT OF ESSENTIAL AMINO ACIDS AND OF REQUIREMENTS FOR THE ESSENTIAL AMINO ACIDS

Amino acid supplementation of foods and feeds is based on the results of over fifty years of research on the nutritional quality of proteins and their constituent amino acids. Much of the fundamental work was carried out by Osborne and Mendel who studied the growth promoting qualities of isolated proteins and recognized that specific amino acids were missing or present in low concentrations in those proteins which did not support adequate growth of the animal. Rose (1937, 1938) divided the amino acids found in proteins into two main categories, the essential and the nonessential amino acids. Essential are those amino acids which the body cannot synthesize at all or not at the rate commensurate with maximum growth and which, therefore, must be supplied in the food. Nonessential is the term applied to those amino acids which the body can synthesize from normal food constituents. On the basis of this classification Rose (1937, 1938) suggested tentative figures for the requirement of the individual essential amino acids by the rat and by the dog (Rose and Rice 1939) and proceeded then to study the human requirements for the amino acids (reviewed by Rose 1957). Rose's amino acid requirement data for the rat, modified only slightly over the years (Rose *et al.* 1949) are still in use today because no major deviations have been found. Minor adjustments will probably be made as amino acid research continues to advance. In the meantime the require-

ments of other species of animals particularly of the growing chick and of the pig have been the subject of continuing research and are dealt with in separate chapters as are discussions on the amino acid requirements of man at different stages of his life. Because of the outstanding importance of such data as guides in proper nutrition, the National Research Council has issued tables containing such figures as part of their bulletins on the Nutrient Requirements of domestic animals and of man. A provisional pattern of amino acid requirements for man has been suggested by the Food and Agriculture Organization of the United Nations (1957).

Our understanding of the role amino acids play in metabolism, their requirements by various species, interrelations between amino acids etc. is the result of the work of many outstanding scientists. It is on the basis of their fundamental work that it has been possible to develop theoretically sound and practical principles for the supplementation of foods and feeds with amino acids. Important portions of this accumulated body of information are reviewed at appropriate places throughout this book. Only a few references to specific background information can be given in this chapter. Some of the problems on amino acid supplementation have been reviewed by Flodin (1953, 1957), Howe (1958), Rosenberg (1957), Scrimshaw *et al.* (1958) and Waddell (1958).

## II THE CONCEPT OF A PROTEIN'S FIRST LIMITING AMINO ACID

Following the discovery of definite requirements for the various essential amino acids, Mitchell and Block (1946) collected amino acid analyses for various foods and proteins from their own and other laboratories and compared the pattern with the tentative requirement figures. They found that there was no protein that conformed in all respects to the published requirement figures. By comparing the ratio of the various amino acids in a protein with the ratio given for the requirements they developed the concept of the first limiting amino acid, the amino acid which is present in the smallest amount in comparison with the required ratio. This basic concept, like that of the specific and quantitative amino acid requirements, has been widely used and has been found to be most useful and entirely valid.

## C GENERAL PRINCIPLE OF AMINO ACID SUPPLEMENTATION OF FOODS AND FEEDS

As a general principle for amino acid supplementation, the first limiting essential amino acid should be added in such an amount that the total of this amino acid in the protein of the diet balances with the amount of the second limiting amino acid and the rest of the protein



mentation i.e. of balancing the first limiting amino acid against the second limiting amino acid in a protein or diet evolved from studies on animal and especially on poultry diets (reviewed by Rosenberg 1957). Apparently this general principle is finding rapid acceptance (Rosenberg and Culik, 1957, Howard *et al*, 1958 Waddell 1958)

#### D PRACTICAL APPLICATION OF THE PRINCIPLE OF AMINO ACID SUPPLEMENTATION OF FOODS AND FEEDS

In order to apply the general principle of amino acid supplementation discussed in the preceding paragraphs it should be necessary only to know the amino acid composition of the food or feed to be supplemented and the amino acid requirement of the species that is to consume the supplemented item. From these data it is easy to identify the first and second limiting amino acid and to calculate the amount of the first limiting amino acid needed to bring it into balance with the second limiting amino acid.

An alternative procedure used frequently in the author's laboratory has the advantage of giving a visual picture of the amino acid pattern of the protein to be supplemented in relation to the amino acid requirement of the species that is to benefit from the supplementation. Actually, the amino acid analysis of the test protein is plotted on a graph on which the amino acids are arranged under a so called "requirement line". The latter serves also as a protein requirement line in the case of the growing chick. Figure 1 shows a picture of the graph actually in use for the latter. This arrangement represents the requirements for protein and for the essential amino acids according to the National Research Council (The histidine requirement is plotted as determined by Rosenberg *et al* 1957). The ordinate is provided with a scale to measure the amounts of amino acids as per cent of diet. Separate graphs have been printed for the requirements of the various species and of man. As an example of the use of these graphs in Fig. 2 the amino acid pattern of bread based on the analyses published by Block and Weiss (1956) is plotted on the graph indicating the amino acid requirements of the growing rat. After the value of each amino acid had been plotted it was obvious that the pattern deviated in many respects from the "ideal" or "balanced" protein represented by the printed requirement line. A new line was then drawn from the origin of the requirement line on the abscissa to the amino acid in shortest supply, lysine. This new line represents the amount of "effective" or "complete" protein in the total protein. In order to determine the approximate amount of the first limiting amino acid, lysine, needed to balance with the second limiting amino acid, the requirement line is turned like the hand of a clock around the origin of



the line on the abscissa until it reaches the second amino acid in shortest supply. This proved to be threonine. The amount of lysine needed can then be read directly from the graph.

The amount of the first limiting amino acid to be used for supplementation and determined by calculation or from the graph should be

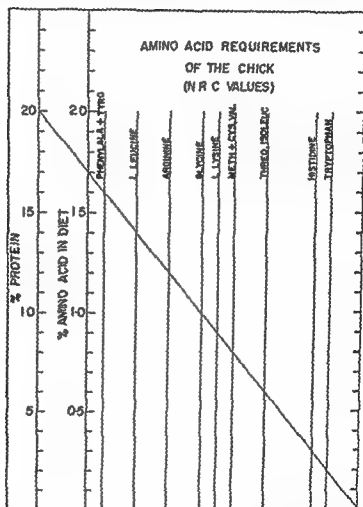


FIG. 1. Amino acid requirement pattern of the growing chick (NRC values)

considered as a tentative figure only. In the present state of our knowledge it is necessary to test this amount experimentally. Especially when a new food or feed item is involved, and if new combinations are tried the experimental test becomes imperative. Such an experiment should be designed around the amount of the first limiting amino acid most likely to yield balance with the second limiting amino acid.

Other experimental groups receiving slightly larger and slightly smaller supplementation as well as the basal diet should be tested (see the examples in Tables II, III, and IV). In our hands the experimen

tally determined value often agreed with the calculated value. Apparent discrepancies can be traced usually to insufficient or incorrect knowledge of the amino acid composition of a particular protein or to inadequate data of the species requirements for one or more of the essential amino acids. Of course there may be other good reasons why in any particular case a calculated value may deviate materially from an experimentally determined one. It may be possible for example that the experimental procedure used to test the supplementation may not be suitable for the purpose. Or the diet used to test a particular protein

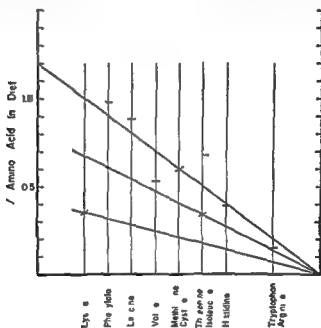


FIG. 2. Amino acid pattern of white bread in relation to the amino acid requirement pattern of the growing rat.

may be unsuitable. For example the diet may not contain enough energy to permit full utilization of the supplemented protein (see III A). Or the diet may contain too much or too little protein (III B). Often however, the problem of amino acid availability may be involved because the nutritional value of a protein is a function not only of the absolute amount of the essential amino acids in the protein but also of the digestibility of the protein and the availability of the amino acids from the native protein for the synthesis of tissue protein (see III D).

Finally there are differences in amino acid requirements due to age and sex (III C) which must be considered when testing for amino acid balance. The amount of any amino acid needed by an organism is in fact dependent upon the requirement of the individual of each species.

This means that all the considerations given to requirement apply also to the amino acid supplementation problem

The practical application of the general principles of amino acid supplementation to proteins and to animal diets is a straightforward process which has created little difficulty. The major factors governing or modifying the amount of the limiting essential amino acid which can be used effectively are believed to be reasonably well understood. For animal feeds there is always the opportunity for directly evaluating the results of amino acid supplementation on the basis of imparted nutritional value as well as on the basis of strict economy.

For man, however, there is no simple procedure for evaluating the effect of an improvement in the nutritional value of a protein or diet. There is, moreover, considerable uncertainty about the amino acid requirements of man. Nevertheless it is reasonable to recommend the use of the best possible protein foods. In the absence of a better procedure it is suggested that the results of animal studies be used as guides for the nutritional evaluation of proteins for the human dietary.

The identity of the first limiting amino acid in many common foods and feeds is known. There is little information, however, as to the extent of this deficiency and of the nutritional value that is obtained after suitable amino acid supplementation. There is a great need for information concerning practically every food item and every feed ingredient. Of course single food items are seldom consumed alone to any appreciable extent. Practical knowledge is required as to the amino acid deficiency of the total protein of a meal so that amino acid supplementation can be considered especially in those countries where acute protein deficiencies are a common occurrence.

In the field of animal feeds mixtures of feedstuffs are practically always used and the problem of devising satisfactory diets is in many ways easier than for man. Here again, however, the amino acid pattern of every item should be known as well as that of the final mixture. Only then will it be possible to assess the benefits attainable by amino acid supplementation of all types of mixed feeds.

#### E. MULTIPLE AMINO ACID SUPPLEMENTATION OF PROTEINS

Systematic studies on multiple amino acid supplementation of proteins have only recently been initiated after the general principles of amino acid supplementation had been recognized. Rosenberg *et al* (1959) have presented evidence which supports the earlier stated (II C) general theory of amino acid supplementation. Accordingly multiple amino acid supplementation should be carried out in such a manner that, in accordance with the requirements of the organism, the sup

plemented amino acids are present in the proper ratio to each other and in balance with the next limiting amino acid or nutrient in the diet

These studies were carried out with rice, corn meal and bread and their supplementation with the first two limiting amino acids was investigated quantitatively. For example, in the case of rice fourteen levels of lysine were tested in various combinations with seven different levels of threonine. The results of rat growth studies were plotted on three dimensional graphs, the two amino acids forming the ordinates and the gain or efficiency of food utilization the third dimension. The graphs obtained resembled pictures of mountain ridges. Mathematical equations were then developed which adequately describe the observed data. The mathematical model chosen is a second degree Taylor Series. For details of this procedure as well as for other important observations made in these studies the reader is referred to the forthcoming papers which describe these experiments.

### III FACTORS AFFECTING AMINO ACID SUPPLEMENTATION OF FOODS AND FEEDS

#### A ENERGY CONTENT OF THE DIET

It has long been realized that a protein can fulfill its nutritional value only if there is sufficient energy in the diet from nonprotein sources to satisfy the requirements of the organism for calories. Recommendations ranging from 11 to 15% of the total calories as protein calories depending upon the quality of the protein and the age of the consumer have been made for man.

That the available energy in a diet can be of critical importance for the successful amino acid supplementation of animal feeds was not realized until recently when a direct relationship between the energy and the protein content was demonstrated in broiler feeds. In poultry nutrition the energy content of a ration is usually expressed in calories of productive energy or of metabolizable energy. Metabolizable energy is the gross food energy minus the energy contained in the excreta which comprises the nondigestible part of the food and the fecal and urinary end products of metabolism. Calories of productive energy are according to Fraps (1946) who determined most of the values in use today, those calories which are stored as fat and flesh, i.e. are available for useful work, maintenance and production. The difference between the two expressions constitutes what is known as the specific dynamic effect. For the purpose of supplementing diets with essential amino acids, productive energy is considered to be the preferred measure. Metabolizable energy values of supplemented and unsupplemented diets are identical but productive energy values change when diets are made more complete by

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amino acid supplementation (Baldini, 1958). When protein energy relationships are considered, ideally only the completely balanced portion of the protein should be calculated as protein and the rest as energy. The amount of complete protein can be expressed easily in terms of the amount of the first limiting amino acid. It was through work on supplementing chick diets with the first limiting amino acid, methionine that the energy-protein relationship was discovered. Using a corn-soybean meal diet little or no response had been obtained in chicks when a small amount of methionine was added to the ration in spite of the fact that

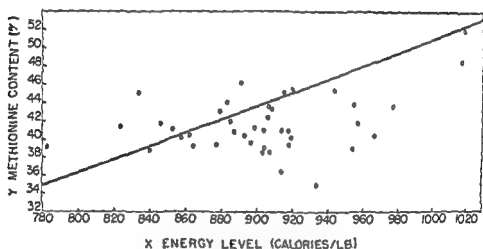


FIG. 3. Methionine energy content of various broiler diets and their response to supplemental DL methionine:  $Y = 0.000736X - 0.2269$ .  $\bigcirc$  = diets improved by DL methionine.

the first limiting amino acid in the diet's protein was methionine. When, however, fat was added to the diet the chicks responded to supplemental dietary methionine with better growth and improved feed efficiency (Rosenberg *et al.*, 1955). This effect was due to increased energy, not to fat per se as was shown later on when carbohydrates were used to replace the fat (Baldini and Rosenberg, 1957). A survey was then made of over fifty different diets which had been tested on chicks for their response to supplementary methionine. The diets were plotted according to their methionine and energy content. A dividing line could then be drawn (Fig. 3) between those diets which responded and those which did not respond to methionine supplementation. Provisionally this line was proposed as the line of ideal balance between the methionine and energy contents of broiler diets at least within the explored area (Baldini and Rosenberg, 1955). The position and slope of this line was then confirmed in a series of specially designed experiments, using isonitrogenous diets and varying the energy content over a 200-calorie range. The combined

results of two such experiments are shown in Fig. 4. A corn-soybean oil meal diet modified to include a substantial amount of permeal was used. This diet was adjusted to three different caloric levels: 800, 900 and 1000 calories of productive energy per pound by interchanging cellulose and fat. As seen in Fig. 4, optimum performance was obtained at different levels of methionine for diets of different caloric content. Results from these and other experiments show that the methionine requirement of the growing male chick may be expressed as per cent of diet in

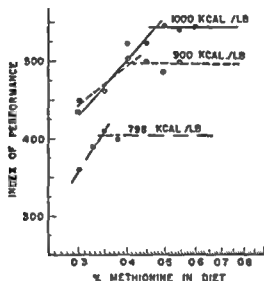


FIG. 4. Methionine requirement of the growing chick consuming 21% protein diets of different energy content.

a straight line function of the energy content of the diet according to formula (1)

$$Y = 0.000736X - 0.2269 \quad (1)$$

in which  $Y$  represents per cent methionine in the diet and  $X$  the productive energy content per pound of diet.

This energy-methionine relationship has been explained on the basis that birds eat primarily to satisfy their energy requirements, although their food intake is governed also by a number of other factors including protein concentration and composition of the protein. When the energy content of a diet is increased birds eat less per unit of gain. Increase in energy content of the diet results therefore in an increase in the efficiency of feed utilization. For example, in a typical chick growth experiment the birds consumed 2.85 gm of feed per gram of gain but after the addition of fat only 2.60 gm of feed was needed for each gram of gain. Assuming that both diets contained 0.45% methionine the birds would



have obtained 12.8 mg methionine per gram of gain on the regular diet but only 11.7 mg on the higher energy diet. If the chicks on the regular diet received the proper amount of methionine for optimum growth, the chicks on the higher energy diet would obtain only about 90% of the optimal amount. As a result they would grow at a slower rate than those which received an adequate amount of methionine. It is obvious then, that care must be exercised to provide the birds in the smaller amount of high energy feed with all the nutrients essential for optimum performance. In other words, as the energy content of a diet increases the amount of essential nutrients, expressed as per cent of diet must also be increased.

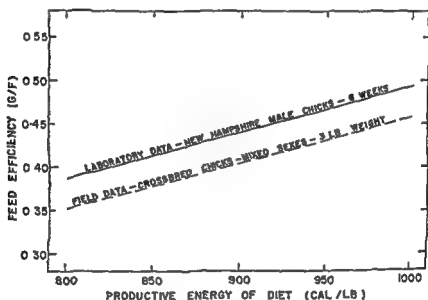


FIG 5 Relationship of energy content of diet to feed efficiency of the chick

The relationship of the energy content of a diet to the efficiency, with which the diet is utilized by the growing chick is shown in Fig 5. While the position of the line depends on various factors such as strain, sex and age of the birds, the slope is considered a good approximation of the feed efficiency energy relationship.

There are of course limitations to these relationships. For example, if an animal is physically unable to consume enough food to satisfy its energy requirements because the ration is bulky and has a low energy content, the relationships obviously do not apply. On the other hand, if the ration's energy content is increased, the amino acid energy relationship ceases to function as soon as one or more of the other amino acids becomes limiting (Rosenberg 1957). This occurs as discussed earlier, when a sufficient amount of the first limiting amino acid has been added to achieve balance with the second limiting amino acid.

This finding of the methionine energy relationship has many implications especially on the considerations concerning nutritional requirements. Obviously the methionine requirement of the chick when expressed as per cent of diet is not as constant as it was believed to be but increases with increasing energy content of the diet. The same principle is believed to apply to all essential amino acids. This has been found to be the case in other experimental studies i.e. for the lysine requirement of the growing rat (Rosenberg and Culik 1953, Rosenberg

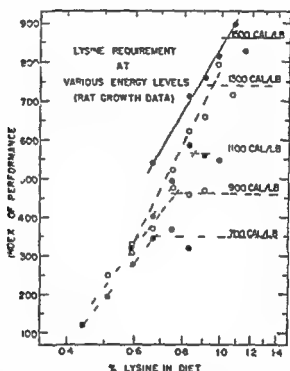


FIG. 6. Lysine requirement of the growing rat consuming 24% protein diets of different energy content.

1957) and of the chick (Williams and Grau 1956, Schwartz *et al.* 1958) and for the methionine requirement of the turkey (Baldini *et al.* 1957).

As another example Fig. 6 shows the results of a study on the lysine requirement of the growing rat on diets of 24% protein and various energy levels ranging from 700 to 1500 calories of productive energy per pound of diet. The protein of the diets consisted of wheat gluten to which all amino acids but lysine were added in such amounts as to bring the level to 15 times the rat's requirement. The only variable in these diets was fat and fiber which were interchanged to obtain the desired energy content. Each diet was supplemented with graded levels of lysine and fed to groups of weanling rats. After 5 weeks the gain and food con-

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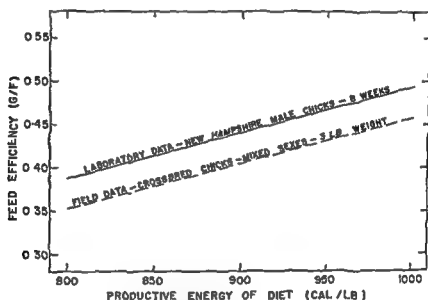


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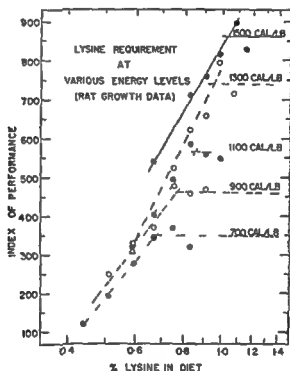


FIG. 6 Lysine requirement of the growing rat consuming 24% protein diets of different energy content

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sumption of each animal on each diet was determined and the index of performance plotted against the total per cent lysine in the diet (Fig 6). Obviously the dietary lysine requirement increased with increasing content of the diet.

Other aspects of caloric intake and nitrogen utilization and of the protein sparing effects of fats are discussed in special chapters of this book.

In animal experiments with low protein diets several investigators noted a slightly smaller amino acid requirement expressed as per cent of diet, when dextrin made up the bulk of the diet than when sucrose was used. The difference was due largely to the smaller intake of the sucrose containing diets, yet it was stated that the effect was not due to a difference in energy content of the diets. The authors attribute the effect to the nature of the carbohydrate. A summary of the data (Harper and Elvehjem 1957) should be consulted if diets of low protein and high sucrose content are to be supplemented with amino acids.

## B PROTEIN CONTENT OF THE DIET

That the amount of protein in the diet may control the amount of the first limiting amino acid that should be added is evident from the discussion on the energy content. When there is a small amount of protein in the diet in relation to the amount of dietary energy, the protein obviously, can be fully supplemented with the first limiting amino acid as described under General Principle of Amino Acid Supplementation (II-C). As the per cent of protein in the diet is increased at constant energy level the point will be reached where the second limiting amino acid will be in balance with the diet's energy content. Upon further increase of protein level it will not be possible to supplement this protein fully with the first limiting amino acid because the amount of energy in the diet will limit the amount of balanced protein that the body can use for protein synthesis. Any excess will be catabolized and used as a source of energy. In accordance with this concept it was found that diets for the growing chick required increasing amounts of the first limiting amino acid methionine as the protein content was increased. This increase in the first limiting amino acid was proportional to the increase in total protein as long as the diet contained sufficient energy to permit the bird to make full use of the balanced portion of the protein for growth and maintenance. If however insufficient energy was available from the nonprotein sources apparently the animal used some of the protein to satisfy its energy requirements. Under such conditions, the noneffective part of the protein, in preference to the balanced portion may be used for energy. This interpretation is based on the finding that

with restricted energy content the requirement for methionine did not increase in proportion to the increase in protein (Rosenberg and Baldini 1957)

This experimentally established relationship may be expressed in a different manner. When the ratio of calories to protein is held constant the amount of amino acids required by the body for best performance and expressed as per cent of protein remains relatively constant when the concentration of protein in the diet is increased. When the ratio of calories to protein is increased the amino acid requirement as per cent of protein increases also. When the ratio of calories to protein decreases the amino acid requirement as per cent of protein decreases.

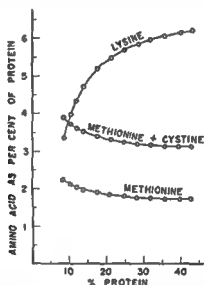


FIG 7 Change in relative amounts of lysine methionine and methionine plus cystine in corn soybean oil meal diets as function of protein content of diet

The effect of a change in protein level is a problem of considerable practical importance as feeds for domestic animals range from 10% protein for the fattening pig to 30% protein for the turkey poult. In the United States most diets are based on corn and soybean oil meal. Corn contains about 8-10% protein while soybean oil meals ranging from 40% to somewhat above 50% protein are available. Each of these two materials has its own pattern of amino acids, lysine being the first limiting amino acid in corn and methionine the first limiting amino acid in soybean oil meal. As these two ingredients are mixed in different proportions to yield diets of different protein concentration the relative amino acid content of the protein in these mixtures changes. This is illustrated in Fig 7 in which per cent protein in the diet is plotted against per cent of amino acid in the protein for lysine methionine and

cially those by Allison (1949, 1951, 1955, 1957) Throughout this book the subject is treated in several different ways. These procedures will therefore not be reviewed here. However, a few problems which are particular to the use of free amino acid will be discussed.

**The Rat Growth Test** It is recommended that any program for the evaluation of amino acid supplementation be started with an evaluation of the effect on the growth of the weanling rat. Diets intended for poultry are perhaps an exception, since they should be checked with the growing chick. Rat feeding experiments are relatively easy to perform and from the results reasonable predictions can be made as to the effect on other species.

The main problem in designing a rat test is to select the level of protein. It must be low enough to permit the measurement of the first limiting amino acid yet the protein should not be stretched too far because otherwise the animals may have neither normal metabolism nor reasonably normal growth. If the complete amino acid pattern of a protein or food is known it would be a good stratagem to select the level of total protein in such a manner that there is enough of the third limiting amino acid for normal growth of the animals but not quite enough of the second limiting amino acid. This would mean, of course, that each protein be tested at a different level which may be objectionable for routine evaluation. In such cases a 10% protein level is usually chosen. Some workers have used lower percentages but the disadvantages of a lower protein diet are usually greater than the advantages. On the other hand low protein diets (6-8%) may have to be used in studies involving the second limiting amino acid. Often especially when the supplementation of low protein food items such as cereals is to be studied there is much to be learned if they be tested as 90% of the diet the remaining 10% to be made up with salts, vitamins and fat if needed. Examples of rice, corn meal and bread evaluated in this manner will be found under V. A comparison of the growth attained from these foods is particularly instructive.

For routine evaluation the use of the male rat only is recommended but in some cases information concerning both sexes may be needed. Usually groups of 6-10 animals are selected per treatment. At weaning (20-23 days of age) the animals are housed individually in screen bottom cages and weekly measurements are made of weight gain and food consumption. The test is carried out over a 3-5 week period. Five week data are routinely obtained in the author's laboratory. Amino acid supplementation is evaluated by adding to the basal diet e.g. to the 90% cereal diet a number of different levels of the amino acid under study. Ideally the levels should be selected in such a manner

that there are at least two levels below the calculated optimum level of supplementation and at least one level above. Three levels below and two above is much to be preferred. The sequence of levels should follow either an arithmetic or a geometric progression. The smallest amount of added amino acid that gives maximum gain and feed efficiency is considered the level at which the first limiting amino acid is in balance with the second limiting amino acid and with the rest of the protein. For practical purposes it is often sufficient to select the level above which there is no further significant increase in performance. Statistical evaluation of the data is desirable.

To assure adequate control of all experimental details a group of rats on a standard diet should be included in each test. A stock diet is often used for this purpose. A special diet based on the Animal Nutrition Research Council's special casein has recently been suggested for this purpose (Chapman *et al.* 1959).

**Protein Efficiency Ratio (PER)** is defined as the ratio between the weight gained and the weight of the protein consumed. For the purpose of comparing various sources of protein for human consumption the PER is usually determined in rat growth experiments run at a 10-15% protein level. PER values are used frequently to express the improvement of the nutritive value of a food attained by amino acid supplementation. A recent survey (Derse 1959) showed that this procedure originated by Osborne and Mendel (1919-1920) is still the method of choice for evaluating protein quality. PER is a valuable tool provided the following two limitations are kept in mind. (1) PER varies according to the level and type of protein in the diet. Sure (1955) for example pointed out that the PER of wheat tended to improve with increasing protein level in the diet, rolled oats yielded essentially the same PER at 7 to 12% protein levels and egg and milk proteins gave decreasing values with increasing protein level. Mitchell (1944) had already called attention to the need of finding the dietary level of a given protein that would yield maximum efficiency as visualized originally by Osborne and Mendel. (2) PER varies with the length of the experimental period. A three week rat growth for example yields a higher PER than a 4 or 5 week experiment. Also the relative decrease in PER from week to week varies with the protein.

**Nitrogen Efficiency Ratio** is defined as the ratio between the weight gained and the weight of the dietary nitrogen consumed. This ratio is then almost identical with the PER but nitrogen values are used rather than protein values. It is more logical to use NER than PER when amino acids are present in the diet because amino acids are not proteins neither physiologically nor chemically and the commonly used factors



to express analytically determined nitrogen in terms of protein do not apply to amino acids

*Feed efficiency* is the efficiency with which a food or feed is utilized for vital functions usually growth and maintenance, or production of meat, milk, eggs etc. It is expressed as the amount of gain per unit of feed. As the number increases the efficiency with which a feed is utilized increases. In practical animal production the reciprocal value is often used. This number obtained on dividing feed by gain, expresses the amount of feed needed to obtain a unit of gain.

When a protein deficient in one particular amino acid is fed to a group of animals and similar groups of animals obtain the same diet supplemented with increasing levels of the deficient amino acid the rate of growth will increase until a maximum growth rate is obtained beyond which no further improvement is attainable. Occasionally, however, it has been observed that maximum feed efficiency may not be obtained at the level of amino acid supplementation found optimum for growth but when a slightly larger amount was added. This is common experience when poultry diets are supplemented with methionine.

*Test for Body Composition* The rat growth test based on weight gains and efficiency of food utilization is, by and large sufficient to appraise the improvement attainable by amino acid supplementation. Actually however, it is possible for two groups of animals to obtain identical gains from two different types of protein yet there may be a considerable difference in the body composition of the two groups of animals. In order to take this factor into consideration the rats at the end of the growth period are killed and their carcasses are analyzed for water, nitrogen and fat. Body composition changes with diet as well as with age. For example the water content of rats in our colony decreases by at least 10% from weaning until maturity and decreases further thereafter. Simultaneously the fat and nitrogen content increase the fat content more so than the nitrogen content. While these changes are very gradual in animals on the stock diet, more rapid changes occur as a result of feeding diets of different composition. As a general rule if the protein content of a diet is reduced below the optimum level or if the protein is markedly unbalanced in respect to its essential amino acid content a deviation from the normal body composition may be expected. There is unfortunately little published information on this subject and on the effect of amino acid supplementation of proteins on carcass composition. Nevertheless there is much practical application to be expected from a more thorough study of this subject particularly for the raising of lean pigs. Possible application to problems of human health may also derive from such studies.

*Efficiency of Nitrogen Retention* This is a procedure for measuring the actual amount of nitrogen retained. In principle a rat growth test is performed and after an appropriate length of time 10 days 3 weeks or 5 weeks the gain and food consumption is recorded and the carcass composition is determined. From the total amount of nitrogen in the carcass is subtracted the total amount of nitrogen present in a similar group of animals at the beginning of the experiment. The difference constitutes the amount of nitrogen retained by the animals. From the amount of feed consumed and the nitrogen content of the feed the amount of nitrogen consumed is calculated. Finally the amount of nitrogen retained by the animals is divided by the amount of nitrogen consumed and the quotient obtained is multiplied by 100. This product represents the per cent of nitrogen retained. This may be expressed in formula (2)

$$\text{Per cent of Nitrogen Retained (\% NR)} = \frac{\text{Carcass N at end of test} - \text{Carcass N at start of test}}{\text{N consumed by animals during test period}} \times 100 \quad (2)$$

In the author's laboratory this method has been used successfully for problems involving amino acid supplementation. An example is given in Table I. Depending upon the purpose of the test the protein level is chosen with the same considerations as discussed under the rat growth test. It is deemed important for example to keep the protein level high enough to avoid starving the test animals for nonessential amino acid nitrogen. Amino acids should in general be added on top of the test protein not replace it. If it should be necessary to determine nitrogen retention at constant dietary nitrogen a source of nonessential nitrogen should be added to the basal diet and this material should then be exchanged on an equal nitrogen basis with the amino acid to be evaluated. This method appears to be identical in principle with the nitrogen retention method used by McCollum and Shukers (McCollum and Simmonds, 1929).

*Net Protein Utilization* Miller and Bender (1955) and Bender and Doell (1957) define Net Protein Utilization by the formula (3)

$$\text{NPU} = \frac{B_F - B_K + I_K}{I_F} \quad (3)$$

where  $B_F$  is the carcass nitrogen of protein fed rats  $B_K$  the carcass nitrogen of rats on a protein free diet  $I_F$  the total dietary nitrogen and  $I_K$  nitrogen from the test diet without the test protein. Net protein utilization according to the authors is the product of "biological value" and digestibility. This method is similar in many respects to the proce

TABLE I  
EFFICIENCY OF NITROGEN RETENTION AND NET PROTEIN UTILIZATION OF BREAD DIETS BASED ON 10 DAYS RAT GROWTH

Diet	Nitrogen in diet (%)	Lysine in diet (%)	Average weight of 6 males (gm)	Average food consumed (gm)	Nitrogen in carcass (%)	% NR <sup>a</sup>	NPU <sup>b</sup>	PER <sup>c</sup>
90% Bread diet	—	—	55.0 <sup>d</sup>	—	2.66	—	—	—
+0.25% L Lysine HCl	2.14	0.30	73.6	84	2.74	31	32	1.31
+0.25% L Lysine HCl + 0.88% DL threonine <sup>e</sup>	2.18	0.50	95.0	109	2.93	55	57	2.57
+0.35% L Lysine HCl + 1.40% DL threonine <sup>e</sup> + 1.10% DL methionine <sup>e</sup>	2.28	0.50	97.8	112	2.77	50	51	2.41
+0.25% L Lysine HCl + 1.0% glycine	2.46	0.58	87.3	81	2.73	47	49	2.17
70% Bread diet	2.36	0.50	93.3	108	2.06	32	33	2.24
+0.25% L Lysine HCl	1.74	0.23	86.3	65	2.95	43	46	1.18
+0.25% L Lysine HCl + 0.88% DL threonine <sup>e</sup>	1.78	0.43	88.3	98	2.62	49	50	2.85
+0.25% L Lysine HCl + 1.00% glycine	1.88	0.43	91.1	100	2.60	49	50	2.76
Protein free diet	1.96	0.43	94.6	105	2.40	39	41	2.90
Stock diet	—	—	50.1	50	2.95	—	—	—
	3.50	1.20	112.3	106	2.08	23	24	2.29

<sup>a</sup> % NR = per cent of nitrogen retained

<sup>b</sup> NPU = net protein utilization

<sup>c</sup> PER = protein efficiency ratio

<sup>d</sup> Data of animals at start of experiment (21 days old)

<sup>e</sup> These high levels of DL threonine and DL methionine were used because they produced a remarkable increase in NPU under the conditions of Benders (1958) experiments

cedure which measures the per cent nitrogen retained. The considerations given the latter procedure in the previous paragraphs should also apply to the determination of the NPU. In the example given in Table I the values obtained for the % nitrogen retained and for NPU are practically identical.

**Nitrogen Balance Index** Among the various nitrogen balance techniques we are particularly impressed by the nitrogen balance index as it gives rise to fewer misinterpretations than any of the other methods. Using this method and six different proteins good correlation was obtained in a collaborative study between the values obtained for the growing rat and man (Allison 1951 1953 1957). Whether or not this good correlation will also hold true for studies involving amino acid supplementation remains to be seen. Nitrogen balance studies on adults are sometimes difficult to interpret as the values obtained depend upon the physiological state of the body, i.e. upon the amount of protein in the body stores. There is a trend toward very rapid adjustment to any change in protein intake by re-establishment of nitrogen equilibrium.

**Protein Utilization Index** used for the evaluation of the protein quality of foods for children is calculated by multiplying weight gains with nitrogen retention in milligram per kilogram body weight (Albani *et al.* 1956). A coefficient of utilization is then obtained by calculating the ratio between the index from the test protein and the index from a reference protein—evaporated milk. "Expression of the bio assay results in this manner has several advantages. First it equalizes disparities between body weight changes and nitrogen retention values which often arise in infants from transposition of body fluid compartments. Secondly it directly relates increments in nitrogenous tissue to qualitative amino acid differences of the test nitrogenous moiety of the diets. And lastly it provides a simple numerical comparison of the test substance with a standard infant food—evaporated milk.

**Performance Index** This is used frequently in commercial broiler production and in other animal production as well as in experimental work when groups of animals are to be compared for weight gain as well as for efficiency of feed utilization. In the simplest form the weight gain is multiplied by the gain over feed ratio (Bird 1955).

**Follow up Procedures** There are many other procedures which have been used for the evaluation of the nutritive value of proteins. No one method can be expected to answer all the questions of the value of amino acid supplementation. Each problem of supplementation is different in some respect from others and to answer that particular phase specific tests may have to be performed or devised. The author's first choice for the study of any problem is as stated above the short range (5 week)

corn meal contained very little protein, 6.75% ( $N \times 6.25$ ), the animals responded readily to an induced amino acid imbalance (Rosenberg, 1959)

TABLE II  
SUPPLEMENTATION OF CORN MEAL WITH L-LYSINE HCl<sup>a</sup>

L-Lysine HCl added to basal 117 (%)	Total lysine in diet (%)	Experiment R 268		Experiment R 304	
		Gain (gm)	Feed Gain	Gain (gm)	Feed Gain
0	0.16	36	10.21	40	7.90
0.0125	0.17	41	9.31	—	—
0.025	0.18	45	7.95	44	7.56
0.05	0.20	51	7.45	55	6.59
0.10	0.24	34	9.91	35	8.04
0.20	0.32	26	14.69	31	8.13

<sup>a</sup> Five week data. Six male rats per treatment.

### B. RICE

This example is taken from a publication of Rosenberg and Culik (1957). Earlier workers had not been able to demonstrate a beneficial effect from the addition of the first limiting amino acid (lysine) alone since they added to the low (7-8%) protein diet, the full requirement of lysine 1.0% of the diet as suggested by Rose (1937) for the growing rat. Thus an imbalance was created resulting in no improvement in the growth rate of the animals. Pecora and Hundley (1951) however had made the important discovery that the combination of lysine plus threonine improved growth considerably in short term rat experiments. According to amino acid calculations however, lysine should be the first limiting amino acid in rice (Mitchell and Block, 1946).

In this example precooked rice was used rather than raw rice in order to test this food in the approximate condition in which it is consumed. The design of the experiment in which the rice was fed as 90% of the diet and the responses at 5 weeks are shown in Table III. Highly sig

TABLE III  
SUPPLEMENTATION OF PRECOOKED RICE WITH LYSINE. EFFECT ON FIVE WEEKS RAT GROWTH<sup>a</sup>

L-Lysine HCl added to basal (%)	Total lysine in diet (%)	Males		Females	
		Gain (gm)	Feed Gain	Gain (gm)	Feed Gain
0.00	0.25	83	5.82	81	5.79
0.05	0.29	106	4.98	114	5.03
0.10	0.33	118	4.81	123	4.68
0.20	0.41	93	4.93	115	4.61

<sup>a</sup> Twenty animals per treatment.

nificant responses to L lysine HCl supplementation were obtained, best gains and efficiency of food utilization being observed at a level of 0.10% of supplementary L lysine HCl for both males and females. Over all growth is roughly twice as good on the optimally supplemented ration as on the inadequately supplemented corn meal ration yet it was only about half as good as that obtained with the fully supplemented bread diet or with the stock diet.

In order to carry the evaluation program beyond the initial stage of the short range rat growth test, a larger number of animals was studied over a half year period. The growth curves for the group of animals on the basal rice diet and on the diet supplemented with 0.1% L lysine HCl

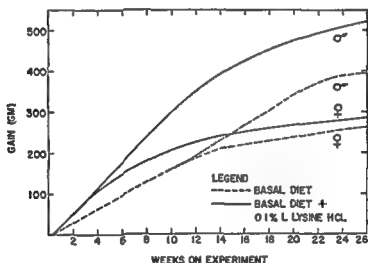


FIG 8 Growth response of rats on precooked rice diet to supplementation with optimum level of L lysine HCl

are reproduced in Fig 8. The important observation here is that the animals consuming the properly supplemented rice diet have grown to essentially the same weight as similar animals do on a stock diet. On the other hand the animals on the unsupplemented diet were distinctly abnormal in size as well as in weight and appearance with rough coats and scaly tails.

### C BREAD

This example is of particular interest because supplementation of various breads has been practiced commercially since 1954. This development is based on extensive studies over a ten year period on the improvement in the nutritional quality of bread by supplementation with lysine, the first limiting amino acid. It was realized from the start that because of differences in digestibility experimental studies should be

carried out with bread not with flour. It was also known from the work of earlier authors that lysine is somewhat subject to destruction by heat. Initially, therefore, a study was undertaken to learn the extent of the destruction of the native lysine in the flour during the baking procedure (Rosenberg and Rohdenburg, 1951). Next these authors (Rosenberg and Rohdenburg, 1952) determined the amount of lysine HCl which when added to commercial white bread, would give optimum response in the 5 week rat growth test. Table IV shows the results of a similar

TABLE IV  
SUPPLEMENTATION OF WHITE BREAD WITH L-LYSINE HCl EFFECT ON FIVE WEEKS  
RAT GROWTH

L-Lysine HCl added to basal (%)	Total lysine in diet (%)	Males		Lysine per gram of gain (mg)	Females		Lysine per gram of gain (mg)
		Gain (gm)	Feed Gain		Gain (gm)	Feed Gain	
0.00	0.33	71	6.00	20	69	6.01	20
0.10	0.41	117	4.58	19	93	4.92	20
0.20	0.49	159	3.70	18	130	3.99	20
0.30	0.57	188	3.30	19	140	3.61	20
0.40	0.65	228	2.97	19	139	3.60	23
0.50	0.73	203	3.04	22	138	3.58	26
0.60	0.81	174	3.24	26	134	3.62	29
0.70	0.89	223	2.93	27	140	3.67	32
0.80	0.97	186	3.13	30	140	3.63	35
Stock diet		218	2.92		137	3.76	

experiment run almost ten years after the first experiment with animals from the same colony and with a commercial white bread containing 5% non fat milk solids (Rosenberg 1959). Addition of 0.4% L-lysine HCl to the 90% air dried bread diet gave maximum response for the males while 0.3% L-lysine HCl was needed by the females. It is to be noted that at these levels of supplementation the animals grew as well as on the stock diet. It seems justified therefore, to repeat the earlier conclusion. These results suggest that as far as rat growth is concerned, the only important amino acid deficiency in commercial bread is lysine.

These investigators proceeded then to a similar growth study extended over 11 months the results of which are seen in Fig. 2. Another group of animals not shown in this graph was fed the stock diet and grew as well as the animals on the bread diet fortified with the largest amount of lysine. Of particular significance are, perhaps the results obtained from the addition of 0.2% lysine. The animals (mixed sex) grew during the period of early growth at an average rate of about 75% of that of a similar group of animals on the stock diet. This disadvantage

was essentially overcome by the time the animals were 6 months old. Animals receiving less than 0.2% lysine supplementation after 25 weeks growth did not attain weights comparable to those receiving the stock diet. It is also obvious that this relatively small addition 0.2% lysine to the basal diet was utilized very effectively. The protein efficiency was much improved over that observed with diets of lower lysine content and not greatly inferior to that found with larger lysine additions.

Breads were then baked from flour to which 0.25 pounds of L lysine HCl had been added per 100 pounds. The growth responses were found similar to those from bread to which the lysine had been added

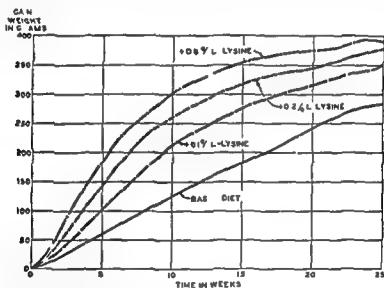


FIG. 9 Twenty five weeks growth response of rats to bread diets containing increasing amounts of supplementary lysine

after baking. On the basis of many experiments of this kind it seemed appropriate to consider 0.25% L lysine HCl as a reasonable addition to flour. The lysine content of the supplemented flour and bread compares favorably with accepted standards (Fig. 10). The flour thus supplemented may also be considered to have been reconstituted to that of whole wheat as far as its amino acid pattern is concerned.

The results of a reproduction and lactation study with rats have recently been published (Culik and Rosenberg 1958) in which the effects of commercial white bread containing 6% milk solids were compared with a similar bread baked from flour supplemented with 0.25% lysine HCl and with the stock diet. The comparison was carried out with groups of animals maintained on their respective diets from weaning until death and bred until fertility ceased. The first litter of the parent generation on each diet was raised to maturity and then mated



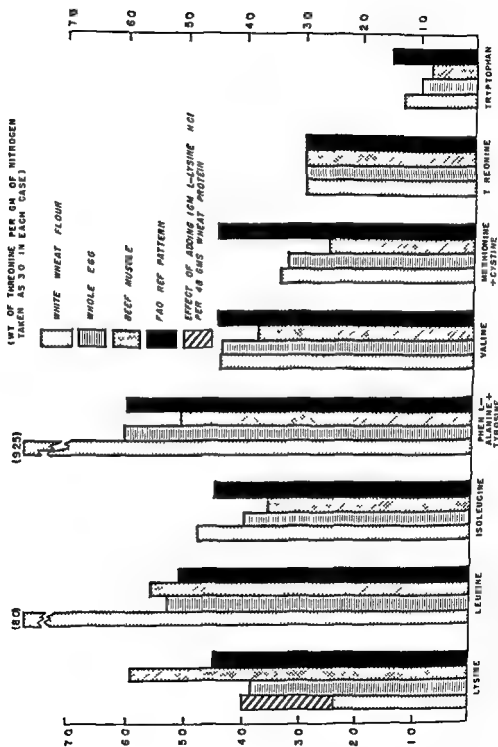


Fig 10 Proportions by weight of essential amino acids in white flour, egg and meat protein compared with FAO reference pattern (based on data from *Home Economics Research, Dept. of Agriculture, Canada*)



and extended. For example, it was shown that regardless of the level of non fat milk solids chosen as addition to commercial bread, lysine is still the first limiting amino acid in bread and the nutritive value of bread can be improved substantially by lysine supplementation.

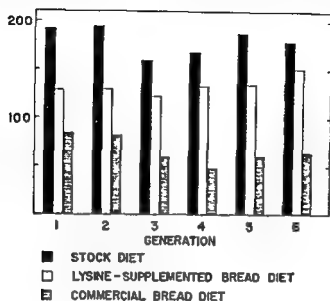


FIG. 12. Growth response of six successive generations of rats on commercial bread diets.

## D. ANIMAL FEEDS BASED ON CORN AND SOYBEAN OIL MEAL

### 1. Broiler Diets

To illustrate the use of methionine and of its hydroxy analog two examples are given. The diets are a 24% protein commercial type 1958 broiler ration and the 21% protein Arkansas performance test diet (Stephenson 1956) the compositions of which are shown in Table V. The results obtained with these diets are seen in Tables VI and VII. Groups of 70 day old crossbred chicks (Vantress males  $\times$  Nichols #12 females) were raised in floor pens for 8 weeks. With diets of this type 8 pound broilers are raised commercially in 8 weeks with a consumption of a little over 6 pounds of feed per bird. This would not be possible without the methionine supplementation which improves both growth and efficiency of feed utilization and is highly economical.

### 2. Turkey Diets

This example has been chosen to illustrate the saving in feedstuffs which can be accomplished by amino acid supplementation (Baldin *et al.*, 1954). Turkey starter rations are usually formulated to contain 18-30% protein. Commercially methionine is now added generally to turkey rations to improve the efficiency. The example in Table VIII

TABLE V  
COMPOSITION OF ANIMAL FEEDS BASED ON CORN AND SOYBEAN OIL MEAL

Components	Corn meal <sup>a</sup> 1958 broiler rations <sup>a</sup>	Arkansas perform- ance test diet <sup>b</sup>	Turkey starter 20% protein	Turkey starter 29% protein	Pig starter
Ground yellow corn	50.67	55.5	60.57	37.57	90.6
Soybean oil meal solv 45% protein	—	—	32.00	53.00	57
Soybean oil meal debulbed 50% protein	26.50	25.3	—	—	—
Alfalfa meal	3.00	2.0	2.50	2.50	—
Fish meal	5.00	4.0	—	—	—
Fish solubles	—	2.0	—	—	—
Meat scraps	3.00	—	—	—	—
Fat	5.00	3.0	—	—	—
Limestone	—	1.4	2.50	2.50	—
Calcium carbonate	—	—	—	—	1.2
Vitamin supplement	Y	Y	Y	Y	Y
Dicalcium phosphate	1.50	2.0	1.25	1.25	0.9
Salt iodized	0.30	0.5	0.45	0.45	0.5
Trace mineral mix	—	—	—	—	0.1
Dried whey	3.00	2.0	—	—	—
Butyl fermentation sol	—	2.0	—	—	—
Dried distillers solubles	2.00	2.0	—	—	—

<sup>a</sup> The commercial formulation of this diet contains 1-2 pounds of DL methionine per ton of feed

<sup>b</sup> The Arkansas performance test diet contains an additional 2 pounds of DL methionine per ton of feed

and extended. For example it was shown that regardless of the level of non fat milk solids chosen as addition to commercial bread, lysine is still the first limiting amino acid in bread and the nutritive value of bread can be improved substantially by lysine supplementation.

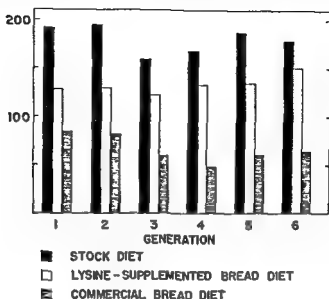


FIG 12 Growth response of six successive generations of rats on commercial bread diets

## D ANIMAL FEEDS BASED ON CORN AND SOYBEAN OIL MEAL

### 1 Broiler Diets

To illustrate the use of methionine and of its hydroxy analog two examples are given. The diets are a 24% protein commercial type 1958 broiler ration and the 21% protein Arkansas performance test diet (Stephenson 1956), the compositions of which are shown in Table V. The results obtained with these diets are seen in Tables VI and VII. Groups of 70 day old crossbred chicks (Vantress males  $\times$  Nichols #12 females) were raised in floor pens for 9 weeks. With diets of this type 3 pound broilers are raised commercially in 8 weeks with a consumption of a little over 6 pounds of feed per bird. This would not be possible without the methionine supplementation which improves both growth and efficiency of feed utilization and is highly economical.

### 2 Turkey Diets

This example has been chosen to illustrate the saving in feedstuffs which can be accomplished by amino acid supplementation (Baldini *et al* 1954). Turkey starter rations are usually formulated to contain 28-30% protein. Commercially methionine is now added generally to turkey rations to improve the efficiency. The example in Table VIII

TABLE V  
COMPOSITION OF ANIMAL FEEDS BASED ON CORN AND SOYBEAN OIL MEAL

Components	Corn meal <sup>a</sup> 1958 broiler rations <sup>b</sup>	Arkansas performance test diet <sup>b</sup>	Turkey starter 20% protein	Turkey starter 28% protein	Pig starter
Ground yellow corn	50.07	55.5	60.57	37.57	90.6
Soybean oil meal solv 45% protein	—	—	32.00	55.00	57
Soybean oil meal debulbed 50% protein	26.50	25.3	—	—	—
Alfalfa meal	3.00	2.0	2.50	2.50	—
Fish meal	5.00	4.0	—	—	—
Fish solubles	—	2.0	—	—	—
Meat scraps	3.00	—	—	—	—
Tat	5.00	3.0	—	—	—
Limestone	—	1.4	2.50	2.50	—
Calcium carbonate	—	—	—	—	1.2
Vitamin supplement	✓	✓	✓	✓	✓
Dicalcium phosphate	1.50	2.0	1.25	1.25	0.9
Salt iodized	0.30	0.5	0.45	0.45	0.5
Trace mineral mix	—	—	—	—	0.1
Dried whey	3.00	2.0	—	—	—
Butyl fermentation sol	—	2.0	—	—	—
Dried distillers solubles	2.00	2.0	—	—	—

<sup>a</sup> The commercial formulation of this diet contains 1-2 pounds of DL methionine per ton of feed

<sup>b</sup> The Arkansas performance test diet contains an additional 2 pounds of DL methionine per ton of feed

shows that a 20% turkey starter ration supplemented with methionine and lysine may give as good a performance as a 28% protein diet supplemented with methionine. The latter combination was not improved by additional supplementation with lysine. Jersey Buff poultz were fed

TABLE VI  
SUPPLEMENTATION OF BROILER RATION WITH DL METHIONINE

DL-Methionine supplementation to 1958 commercial type broiler ration <sup>a</sup> (%)	Nine week gain of broilers (gm)	Feed Gain	Index of performance
0	1536	2.45	627
0.05	1564	2.30	680
0.10	1596	2.30	691
0.15	1594	2.30	693

<sup>a</sup> When this diet is mixed commercially DL methionine is added at the rate of 1-2 pounds per ton

TABLE VII  
THE METHIONINE RESPONSE IN THE ARKANSAS PERFORMANCE TEST DIET

Supplementation to test diet	Weight gain of broilers at 9 weeks (gm)	Feed Gain	Index of performance
—	1562	2.51	630
0.05% DL Methionine	1658	2.39	694
0.05% Hydan <sup>a</sup>	1640	2.38	692

<sup>a</sup> Hydan — Du Pont's calcium methionine hydroxy analog

TABLE VIII  
SUPPLEMENTATION OF TURKEY STARTER DIET WITH AMINO ACIDS

Treatment	Average 6 week gain (gm)	Feed Gain
20% Protein basal	671	2.30
20% Protein basal + 0.30% L lysine HCl	785	2.17
20% Protein basal + 0.20% DL methionine	749	2.16
20% Protein basal + 0.20% DL methionine + 0.30% lysine HCl	807	2.17
28% Protein basal	764	2.33
28% Protein basal + 0.20% DL methionine	812	2.27

The diets shown in Table V. The saving in feed possible by reduction of protein level to 20-22% and supplementation with both lysine and methionine has been confirmed (Fisher *et al*, 1956). There are great opportunities for further improvements in turkey diets by increasing the productive energy content, lowering the protein level and careful amino

acid supplementation (Baldini *et al* 1957) This may become the first practical use of two amino acids when the price of lysine should make this more economically attractive

### 3 Pig Diets

Catron *et al* (1953) have published the results of a study in which pigs were given a 12% corn soybean oil meal diet (Table V) supplemented with graded doses of lysine The results shown in Table IX,

TABLE IX  
SUPPLEMENTATION OF PIG DIET WITH L LYSINE HCl<sup>a</sup>

L Lysine HCl as supplement to diet (%)	Daily gain (pounds)	Feed Gain
0	1.07	3.74
0.05	1.11	3.22
0.10	1.21	3.01
0.15	1.18	3.14

<sup>a</sup> Poland China × Landrace × Duroc pigs self fed from an average of 22.8 pounds to 100 pounds

illustrate the point made earlier that corn soybean oil meal mixtures tend to become deficient in lysine as the protein level is lowered Best response was obtained with 0.1% supplementary L lysine HCl These data are of considerable practical importance as many farmers in the corn belt feed their pigs probably no better diets than the unsupplemented diet used in this test

## VI CONCLUSION

It has been shown that the amount of "effective" "balanced" or "complete" protein in any food or feed can be increased by appropriate supplementation with the first limiting amino acid The amount of supplementation that can be used effectively is governed mainly by the concentration of the second limiting amino acid present in the food or feed and available to the organism Proper supplementation is achieved when the amount of the first is in balance with the amount of the second limiting amino acid and with the rest of the protein according to the needs of the species All other nutrients must of course be present in the diet to assure full utilization of the balanced portion of the protein These theoretically sound principles are used presently by the highly competitive feed industry for formulation of various poultry diets Little use has been made of the opportunities for the human dietary with the exception of providing a superior bread which is commercially available in some areas of this country Further development awaits the demonstration that the improvement of basic foodstuffs such as rice and corn



can be of benefit. As these data become available amino acid supplementation will be used wherever needed to improve the health and well being of people throughout the world.

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## CHAPTER 14

# Protein and Amino Acid Requirements of Children

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The estimation of the protein and individual amino acid requirements of man constitutes a recently opened field of biochemical inquiry. Its advance is hampered by the incomplete understanding of the protein needs of the human at various life periods. A survey of the literature reveals that despite many efforts (Albanese 1950, Rose 1949, Leverton 1953) there still exists a startling insufficiency of data upon which studies of specific amino acid requirements from infancy to old age must be based and points out the need for a further systematic filling in of these gaps.

### I THE FIRST YEAR OF LIFE

#### A PROTEIN NEEDS

In attempting to assess the protein needs of the growing child we have to judge protein adequacy of the diet by the rate of nitrogen retention and weight change. Unfortunately exact values for normal retention at different ages are not on hand. Wide variations of 100% or more are encountered in the data recorded in the literature. These are due to several factors chief among which is the previous nutritional state of the subject. During convalescence an undernourished child will retain extraordinarily large amounts of protein. Apart from preceding illness

diets vary in their ability to induce storage of reserve protein. If the experimental diet is more conducive to this than the preceding diet, high nitrogen retention will be observed, and vice versa. It is usually accepted that the increased nitrogen retention brought about by increasing the nitrogen intake is a relatively temporary affair lasting a matter of weeks only, after which the original retention level is resumed. Nevertheless, observations have been presented by Nelson (1930) that some increase of nitrogen retention continues indefinitely. These serious limitations should always be borne in mind when evaluating the nutritional characteristics of experimental diets from nitrogen retention data of growing subjects.

Since the amino acid composition (Macy *et al.*, 1953) and caloric distribution of various milks are reasonably well established, the amino acid needs of the premature and full term infants through the first year of life can be determined approximately from their milk intake.

### 1. Prematures

The nitrogen requirements of premature infants younger than 3 weeks (1600 gm) have not been studied. Several earlier investigations (Smith 1945, Gordon *et al.* 1937) have provided evidence that 1-2 months old prematures retain about 250 mg of nitrogen per kilogram per day on an intake of 360-500 mg of milk protein nitrogen per kilogram per day. Gordon also found that, at the comparable level of feeding for premature infants, no difference could be detected between the nitrogen retention of infants receiving modified cow's milk and that of infants receiving human milk for periods as long as 2 weeks. The plotting of Gordon's data as shown in Fig. 1 indicates that nitrogen intakes of less than 450 mg of nitrogen and higher than 500 mg of nitrogen per kilogram of body weight, result in retentions which are respectively below or above the mean, thereby suggesting that 475 mg of milk protein nitrogen per kilogram of body weight per day adequately fulfills the protein requirements of the premature infant. Inasmuch as the mixtures of cow's milk were modified by the addition of Dextrinmaltose, olive oil and water so that they approximated the human milk in protein, fat, carbohydrate and fluid content, the only remaining variable resided in the amino acid composition of the protein moiety. Since human milk used in these experiments contained an average 1.44 gm protein per 100 ml, or 230 mg of nitrogen per 100 ml, it would be necessary to feed 206 ml of human milk to attain the apparent required intake of 475 mg of nitrogen per kilogram per day. Calculations from these figures and the data on amino acid content of human and cow's milk reported by Williamson (1944) and by Soupart *et al.* (1954), make it possible to

estimate the individual amino acid intake of the premature infant per kilogram per day (Table I). These values of course do not represent minimal quantities but give a safe estimate of the range of the needs of the individual amino acids.

In 1947 Gordon *et al* reported studies on 122 premature infants with weights varying from 1000 to 2000 gm, using three diets each giving 120 calories per kilogram per day plus vitamins A, D, and C.

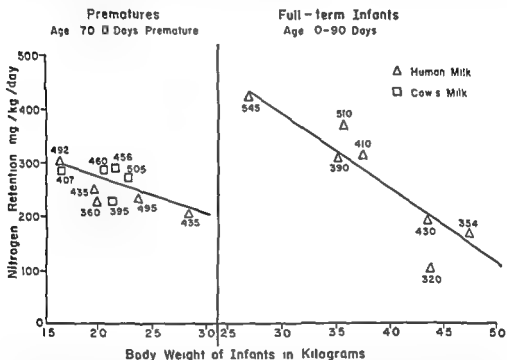


FIG 1 Relation of nitrogen retention to milk protein nitrogen intake and body weight of premature and full term infants. The chart was constructed from the data of Gordon *et al* (1937) on prematures and the data given by Czerny and Keller (1925) on the full term infants. They represent feedings of modified cow's milk and the feeding of human milk. The figures inscribed about these signs denote milligrams of nitrogen intake per day per kilogram of body weight (Albanese 1947).

Diet No 1 consisted of breast milk in which 7% of the total calories were derived from protein. Diet No 2 was a modified evaporated milk formula in which the protein furnished 16% of the total calories. Diet No 3 was a half skimmed powdered cow's milk formula in which 20% of the total calories were derived from protein. Their results indicate that the infants fed cow's milk formula showed a more rapid rate of gain than those fed breast milk (Fig 2).

Many clinical studies have been reported which amply support Gordon's observations at the practical level. Adams (1945) fed 56 pre-

matures on evaporated milk and water, equal parts (3.5% protein), with good results indicated by adequate weight gain. Smellie (1948) found that marasmic and premature infants gained weight normally and

TABLE I

DAILY AMINO ACID INTAKE FOR THE FIRST YEAR OF LIFE CALCULATED FROM MILK PROTEIN REQUIREMENTS NECESSARY FOR OPTIMAL NITROGEN RETENTION<sup>a</sup>

Amino acid	Prematures (70-90 days premature) Modified cow's milk	Full term infants	
		(0-90 days) Human milk	(3-12 months) Modified cow's milk
	(mg amino acid/kg body weight)		
Alanine	76	59	59
Glycine	12	10	14
Proline	256	134	300
Glutamic acid	695	384	820
Aspartic acid	170	194	199
Serine	164	115	192
Threonine	156	105	183
Leucine	504	380	590
Isoleucine	170	125	199
Valine	176	111	203
Cystine	29	69	34
Methionine	103	49	120
Tyrosine	176	122	204
Phenylalanine	188	129	210
Histidine	66	42	78
Arginine	131	112	154
Lysine	205	157	240
Tryptophan	44	52	58

<sup>a</sup> These figures are undoubtedly above the minimum requirements for the particular amino acid in question and it might be possible to reduce the given values for nonessential amino acids to zero. It is obvious from the available data that provided all the requirements for essential amino acids are met the remainder necessary to make up the total required quantity could be distributed among different types of amino acids with wide possible variations all of which might be of equal nutritional value. Of course we must always bear in mind the possibility that the nonessential amino acids may become essential by virtue of a specific nutritional need or by their sparing action of some essential amino acid e.g. the cystine-methionine or the tyrosine-phenylalanine relationship (Albanese 1947).

showed consistently higher serum protein levels when either breast milk or cow's milk formulas were supplemented with 2.2% amino acids in the form of casein hydrolyzate. Powers (1948) reported that feeble premature infants did well clinically on cooked cow's milk formulas with lower fat and higher protein and carbohydrate than breast milk sug-





the first month, and 1.7 times the control group in the second half of the third month. Rothe Meyer (1949a) compared prematures (14-42 days old) fed either a half skimmed citric acid milk formula or breast milk, and found that the infants gained faster on the modified cow's milk mixture. He made the observation, however, that the blood urea nitrogen was higher in this group suggesting a possible increased functional strain. Further studies by Rothe Meyer (1949b) in prematures on breast milk showed serum protein and albumin levels to be less than those found in prematures fed cow's milk formulas. Schreier (1949) observed that premature infants fed breast milk with a protein hydrolyzate supplement showed a definite increase in nitrogen retention in spite of increased excretion of fecal and urinary nitrogen.

In 1950 Young *et al.* studied 203 prematures, weighing over 1600 gm each from birth to 8 weeks of age. A routine evaporated milk formula was fortified by an enzymatic hydrolyzate of casein which supplied 55-60% of the protein intake. Weight gain in the first 8 weeks of life was approximately the same for supplemented and control groups. Satisfactory growth was obtained in those receiving 7 gm of protein and 128 calories per kilogram per day. However, those receiving formulas of lower caloric value, 90-126 calories per kilogram per day, did not gain as rapidly nor could the growth rate be increased by raising their protein intake from 3 gm to 5 gm per kilogram per day. It was suggested that this poor response of the latter group might be due to the use of proteins for energy production rather than for growth. Landucci (1950) also reported that 20 healthy prematures who were not gaining weight satisfactorily on the usual routine, did better after adding 1.5-5.0 gm protein hydrolyzate.

Schneegans (1951) reported that in premature or weak infants, supplementing the breast milk feeding with a mixture of 0.5-1.0% amino acids improved the weight gain, and that amounts above 1% caused gastrointestinal upsets. Feinstein and Smith (1951) published metabolic studies in prematures on cow's milk formulas using supplements of whole protein against hydrolyzed protein. Their results disclosed no appreciable difference in nitrogen absorption or weight gain indicating no impairment of protein digestion in the premature infant. Sisson and co-workers (1951) carried out balance studies on premature infants involving variations in nitrogen, fat, calcium, phosphorus, and iron intake with observations on hemoglobin, blood proteins, growth, weight change and stools. Their results showed that meat protein is retained and utilized as well as milk proteins but needs supplementation with calcium, phosphorus and minerals.

In the following year, Andelman and associates (1952) reported their investigations on premature infants fed a modified milk formula modified milk formula plus meat and breast milk. The infants were given the same number of calories per day and all had comparable hemoglobin levels at 12 weeks of age. The breast milk group showed a slightly increased rate of growth. Childs (1952) reported that the urinary excretion of amino acid nitrogen in premature and young infants is 0.45 mg per kilogram per day, as contrasted to 1.8 mg per kilogram per day in older children. De La Villa and Rodriguez (1952) treated premature infants with amino acid mixtures both by oral and parenteral route. These workers felt that after the period of initial weight loss the supplemented group showed an improved weight gain and that the initially smaller babies gave better results. The parenteral route of administration was preferred in order to avoid irritation of the gastrointestinal tract. Levine and Dann (1952) restated their earlier view on the greater daily protein needs of premature infants (Table II). They emphasized again the greater needs of the premature infant for calcium and phosphorus which are met better by the higher mineral content of cow's milk. Properly modified cow's milk in his opinion is superior to human milk in the feeding of premature infants under controlled conditions of hospital care. In this connection it is interesting to note that Ferreira and his associates (1953) observed that premature infants fed breast milk supplemented by a 1% mixture of amino acids showed an improved growth curve. Chromatographic analyses showed a rise of the amino acid concentration in the blood. LoBianco (1953) studied 48 infants and found also by means of paper chromatography that the premature showed an increased nitrogen retention when compared with the full term infant. He also noted that formula fed infants excreted more amino acids than the breast fed infants.

Berfenstam and co workers (1955) compared the digestive capacity of premature infants 6-44 days of age with that of similar aged full term infants. They instilled into the stomach fat free breast milk or skimmed cow's milk, measured the degree of digestion in terms of the per cent amino nitrogen in the total stomach nitrogen and found indications of superior digestion of the cow's milk protein. Studies of kidney function in the premature show a reduced inulin clearance and PAH clearance with a relatively low serum bicarbonate and high serum chloride thought to be due to an immature kidney. This led Kagan and associates (1955) to the contention that the increased ash content of the high protein cow's milk formulas may produce water retention thereby giving an apparent weight gain in prematures of 1000 to 2000 gm for the first month of life which is not all due to an increase in body tissue.

TABLE II  
RECOMMENDED DAILY ALLOWANCES FOR PROTEIN EXPANDED FOR THE GROWING PERIOD<sup>a</sup>

Subject	Age	Protein in grams <sup>b</sup>			% of Dietary calories average
		Total 1	Per kg 2	Per lb 3	
Premature <sup>c</sup>	1 week to 1 month		6.0-4.4	2.7-2.0	17
Premature <sup>d</sup>	1 week to 1 month	4-3	5.0-4.4	2.3-2.0	15
Premature	1 to 3 months	per kg	4.4-3.3	2.0-1.5	13
Full term	2 days to 3 months		4.4-3.3	2.0-1.5	13
All infants	4 months to 1 year		4.0-3.0	1.8-1.4	13
Toddlers	1 through 3 years	40	(4.2-2.9)	(1.9-1.3)	(13)
Preschool	4 through 6 years	50	(3.3-2.5)	(1.5-1.1)	(13)
School	7 through 9 years	60	(2.6-2.1)	(1.2-1.0)	(12)
Youths female	10 through 12 years	70	(2.2-1.8)	(1.0-0.8)	(11)
Youths male	13 through 15 years	80	(1.8-1.5)	(0.8-0.7)	(11)
Youths female	13 through 15 years	85	(2.0-1.7)	(0.9-0.8)	(11)
Youths male	16 through 20 years	75	(1.6-1.4)	(0.7-0.6)	(13)
	16 through 20 years	100	(2.1-1.7)	(1.0-0.8)	(11)

<sup>a</sup> Levine (1945)

<sup>b</sup> Column 1 gives the allowances recommended by the Food and Nutrition Board columns 2 and 3 the suggested modifications for infants. The figures in parentheses in these columns beyond 1 year represent the total allowances in the original recommendations (column 1) per unit of body weight on the basis of average weights for age groups derived from the tables of Baldwin and Wood.

<sup>c</sup> Premature infants weighing less than 2 000 gm (4 lb 6 oz)

<sup>d</sup> Premature infants weighing 2 000 gm and over

Unfortunately data on the mineral components of the ash content of the formulas tested critical to this concept, are lacking.

From an extensive review of the literature, Higgons and collaborators (1957) found that there is considerable evidence in favor of the opinion that premature infants show a superior pattern of growth and development when fed a high protein relatively low fat, cow's milk formula rather than breast milk. It has been shown that the premature utilizes cow's milk protein in amounts up to 11 gm per kilogram per day, as well as it does protein in human milk. On the other hand, the evidence indicates that the premature handles fats with much less efficiency, and that the fat intake should not exceed 2 gm per kilogram per day. Carbohydrates are admittedly handled well by either the premature or the full term infant. The higher content of calcium and phosphorus in the cow's milk formulas is beneficial to the premature's need for rapid growth of bone.

## 2 Full term Infants

Mathematical analyses of nitrogen balance data (Czerny and Keller 1925) for the first 90 days of life of the full term infant of normal birth weight (Fig 1) indicates that an intake of 400 mg of human milk nitrogen is required for normal nitrogen retention which again appears to be a function of weight rather than age. Comparison of the nitrogen retention curves of the two groups reveals a greater gradient for the full term infant than for the premature infant suggesting a more efficient nitrogen utilization in the premature at the usual levels of protein intake.

Numerous determinations have been made of the protein nitrogen needs of infants 3-12 months of age employing human milk and a variety of modifications of cow's milk. Some of the available data have been recalculated to a uniform base and are listed in Table III. The nitrogen retention values fail to indicate any nutritional advantages of the different modifications. Although it is not evident from Table III examination of the original data reveals that the quantity of nitrogen stored tends to increase with well tolerated increases in milk intake. This phenomenon is not only apparent for short periods of time but has also been shown by Nelson (1930) to lead to the development of larger babies if continued for long periods of time. No doubt the maximal retention which can be effected in this fashion must be limited in magnitude and duration. Jeans and Stearns (1933) observed that the retentions of nitrogen were larger below 20 weeks of age in the group fed fresh milk and between 20 and 35 weeks in the group fed evaporated milk. A graphic comparison of some of these data with those obtained by Albanese (1953a) is shown in Fig 3. The nitrogen retention and body

TABLE III  
RELATION OF MILK PROTEIN NITROGEN INTAKE TO NITROGEN RETENTION OF THE INFANT

Age in weeks	Dextrimaltose modified cow's milk <sup>a</sup>				Acidified undiluted cow's milk <sup>b</sup>				Acidified diluted evaporated milk <sup>c</sup>			
	Nitrogen				Nitrogen				Nitrogen			
	No of subjects	Body weight (kg)	Intake (mg/kg)	Retention (mg/kg)	No of subjects	Body weight (kg)	Intake (mg/kg)	Retention (mg/kg)	No of subjects	Body weight (kg)	Intake (mg/kg)	Retention (mg/kg)
5-10	1	5.40	497	113	5	5.18	688	211	7	5.44	640	179
10-15	8	5.83	535	168	16	6.26	652	185	13	6.24	599	143
15-20	12	6.66	525	136	15	7.26	580	170	15	7.06	589	147
20-25	6	7.70	525	149	9	8.07	581	146	13	8.07	579	193
25-30	5	7.80	565	160	8	8.39	600	147	13	8.28	593	186
30-35	2	9.77	485	86	6	9.09	582	154	8	8.64	604	201
35-40					5	9.59	550	144	9	9.65	530	113
40-45					7	10.49	530	123	8	9.80	495	139

<sup>a</sup> Daniels and Heyman (1929)

<sup>b</sup> Nelson (1930)

<sup>c</sup> Jeans and Stearns (1933)

weight effects of severe reductions in milk protein intake of infants observed by Koye and associates (1954) are also to be noted

With due consideration of the restrictions on the significance of nitrogen retention values, it appears from the available data that 500-600 mg of cow's milk protein nitrogen per kilogram of body weight should be fed infants of this age group. This is somewhat higher than the

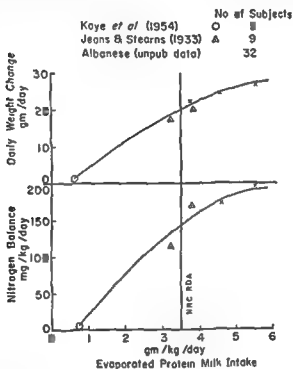


FIG 3 The effect of evaporated milk protein intake on nitrogen balance and daily weight change of infants

average 475 mg of protein nitrogen fed the prematures and newborns by Gordon *et al* (1937). Unpublished studies from our laboratory show that a protein intake of less than 50 gm per kilogram per day is not consistent with normal body weight gain in sick or convalescent infants (Fig 4).

## B PROTEIN QUALITY AND NEEDS

### 1 Species Differences

Numerous studies have been reported on the effect of protein quality on the protein needs of the full term infant. Many of these investigations have concerned themselves with the nutritional efficacy of the protein components of cow's milk and breast milk with and without various nitrogenous supplements.

Despite the quantitative differences in composition of the milk of different species, man and most farm and laboratory animals generally thrive on cow's milk. Some interchanges, however, have not proved completely satisfactory. While kids make the same growth per calorie on cows as on goat's milk (Gamble *et al*, 1939), calves are said to react unfavorably to goat's milk, and while foals are said to react unfavorably to undiluted cow's milk, they thrive on undiluted goat's milk, even

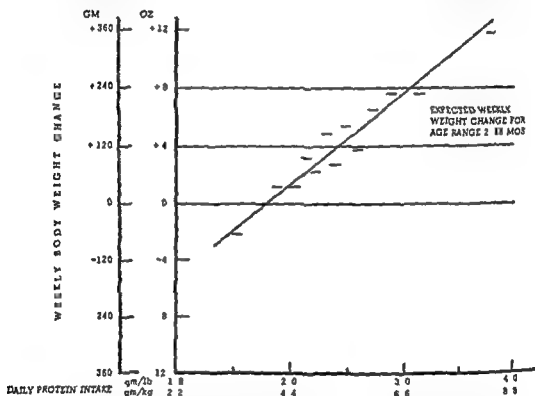


FIG 4 The effect of protein intake on weight change of convalescent infants. This diagrammatic representation is based on weekly measurements made on 12 infants (2-15 months) for periods of 7 to 26 weeks.

though goat's and cow's milk appear to have the same quantitative composition. Fresh cow's milk is not satisfactory for feeding very young puppies partly at least because the concentration of protein, fat, and minerals in cow's milk is so much lower and the sugar so much higher than it is in bitch's milk (Earle 1939). Young kittens have been observed to reject and thrive poorly on fresh milk but accept and grow well on evaporated milk which contains proteins in double the amount of fresh milk (J. O. Albanese, 1950). Scott and Norris (1949) have shown that human milk is entirely unsuitable as a food for the rat.

In addition to the limiting nutritional factors which may arise from protein concentration and amino acid composition of the proteins the

poor biological value of various milks in diverse species may be associated with differences in permeability of the digestive tract lining to proteins of heterologous milk and consequent allergic reactions (Ratner, 1935)

A most interesting discussion of some nutritional differences of various adult mammals can be found in Williams monograph "Appraisal of Human Diets by Animal Experiments" (1947) Some pertinent examples are worth mentioning here

Dr Jet C Winters reported at this conference that the growth of rats maintained on the so called Average American Diet approached normal they mated at maturity but the females were often sterile Their young frequently failed to survive and those that did so were usually stunted at weaning There were many stillbirths and many young were eaten by the mothers Lactation appeared poor A companion experiment with chicks indicated serious defects of such human diets for the growth of that species also

Dr H E Robinson reported at this conference that considerable mammalian species variation in nutritional requirements for reproduction prevails After dozens if not hundreds of experiments he concluded that the rat is not a satisfactory test animal for canned dog or cat food nor is the dog a satisfactory test animal for the cat food

Obviously the physiological bases for the nutritional differences of mammals are multiple Prime consideration must be given to variations in the net dietary energy required for growth of the young and subsequent maintenance of the adult structure variations in the rate of growth of organs and ultimate relative size of these organs and finally the presence of special structures e.g. hair horns mammary glands

## 2 Breast Milk

Although some subjective clinical investigations (Grulee et al 1935) have supported the claim that human breast milk has nutritive advantages over other types of infant foods the available objective observations fail to support this contention Stevenson (1947 1949) recorded that infants fed cow's milk formula showed an increase in percentage of nitrogen content of the body at a rate about equal to the fetal rate whereas breast milk fed infants held the percentage of body nitrogen at about birth levels This effect was attributed to the higher protein value of cow's milk and it was concluded that the usual artificial feeding procedures are adequate Jeans (1950) stated that a positive nitrogen retention must represent an increase in tissue mass as protein is not stored elsewhere in the body Most of the increase in tissue mass is in the form of muscle He observed that babies on cow's milk formulas of relatively



high protein content developed 25% more muscle mass than did breast-fed babies

Ross (1951) published chromatographic studies of the feces of 12 nursing infants and 14 infants on cow's milk formulas. He noted that the breast fed infants showed more total nitrogen and free amino acid in the stool with alanine being predominant, whereas the cow's milk group showed a predominance of valine and lysine. He also found that dilution of cow's milk formulas gave an amino acid pattern similar to that of breast milk. He further reported that, when the gastrointestinal tract is sterilized by the use of antibiotics, more amino acids appear in the stool. Reinlein and Geering (1950) observed that breast fed babies showing nutritional disturbances improved clinically after the addition of a supplement of beef serum which afforded 1.5-2.0% protein.

Evidence is also on hand which supports the view that, even on the most liberal estimates, breast milk alone provides a very meager margin of nutritional safety during the early months of life, and that it does not provide an adequate quantity of some essential amino acids in the latter part of the first year if fed as the principal source of protein (Albanese, 1950). The most impressive of this evidence is that provided by the investigations of Su and Liang (1940) on the growth and development of Chinese infants in different nutritional environments. A graphic analysis of 322 refugee infants on Wetzel Baby Grids shows that a soybean supplement was very effective in preventing retrogradation of growth which befell (Hou, 1939) those who received little if any dietary supplements to breast feeding during the latter part of infancy (Fig. 5).

In recent years teleological reasoning, with an incomprehensible neglect of the facts, has led some to propose that the protein allowances for infants be reduced from 3.5 gm. per kilogram per day, currently recommended by the Food and Nutrition Board to 1.5-2.0 gm. per kilogram per day. These latter figures are based on estimated intakes of protein by breast fed infants. A little thought will reveal that as yet no accurate means have been devised for determining the quantity or quality of the nutrients derived by the infant directly from the breast. Holt (1957) has pointed out that the evidence for this proposal is largely indirect, namely, intakes of milk have been measured by weight gains after feeding, and average analyses of breast milk have been used to calculate the intake. This was done by a number of different observers back in the last century, the most extensive work being done by Emil Feer (1896) in Switzerland. Further, it is well known that the quantity of protein in breast milk may vary from 0.7 to 2.0%. Changes in protein content also occur during the course of a single feeding (Holt and McIntosh, 1940). Data derived

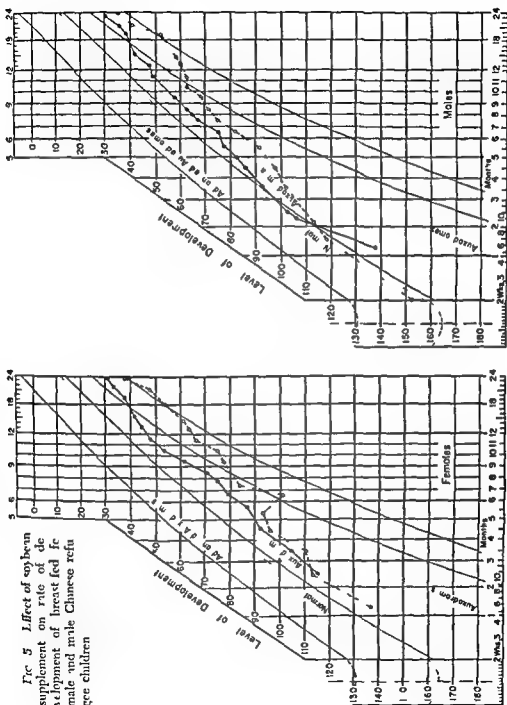


FIG. 5. Effect of soybean supplement on rate of development of breast-fed female and male Chinese refugee children.

Glaser and Johnstone (1952) observed that newborn and young infants, showing evidence of cow's milk allergy, responded better clinically to a feeding containing meat protein than they did to a feeding with soybean protein. Rowe and Rowe (1954) studied 16 children from 2 to 18 months of age with various manifestations of allergy involving the skin, gastrointestinal tract, or respiratory tract. The infants were treated with a meat base formula containing strained meat, soy oil, sugar, potato starch, calcium carbonate salt, and water—equivalent to cow's milk—for periods varying from 2 to 27 months, with clinical improvement in most of the cases. Ziegler (1953) reported that lean meat formulas properly supplemented with calcium, phosphorus, and other minerals are adequate as milk substitutes. In connection with the foregoing observations, it should be noted that Lyman and Kuken (1949) and Block and Bolling (1951) have noted a relative deficiency of lysine and other amino acids in cow's milk versus muscle proteins.

### 5 Vegetable Proteins

The importance and need for studies on the nutritive value of vegetable proteins in the dietary of children can be readily appreciated when it is recalled that seed proteins of legumes and grains constitute the principal and sometimes the only, dietary protein of the major segment of the world's population. Although many survey and clinical reports on this subject are available, relatively few controlled investigations have been done.

Because of ready availability and widespread use, the nutritive value of soybean proteins has been intensively studied. The efficacy of soybean flour as an infant food supplement has been amply demonstrated by Stearns (1933) and others (Payne and Stuart, 1944) in this country and, as previously mentioned by Hou (1939) in China. The hypoallergenic characteristics of soybean preparations have also led to their use in the complete feeding of infants suffering from allergy to milk proteins. Glaser and Johnstone (1953) have found these products clinically useful in the management of infant allergies. However, adequate growth and nitrogen balance data are lacking for an objective evaluation of the nutritive characteristics of these commercial products. In our preliminary efforts, the high stool bulk and poor acceptance encountered with the feeding of the now commercially available soybean products precluded completion of the metabolic balance studies necessary to provide such data.

Corn meal is employed as a supplementary and sometimes as the only infant food in many parts of the world—especially in Latin America. The lysine and tryptophan deficiencies of corn protein are well known.

from the early rat studies of Osborne and Mendel (1911). In the rat growth evidences of these deficiencies were remedied by additions of calculated amounts of the 2 essential amino acids. Albanese and associates (1949b) reported their findings on the biological value of a corn protein in normal male infants. By means of controlled studies they found that nitrogen retention and weight gain of infants maintained on a synthetic diet in which tryptophan and lysine supplemented commercial zein (Protein 3223) constituted the principal source of nitrogen were inferior by reason of the poor digestibility of this zein to those obtained on a casein diet fed at the same fluid caloric distribution and nitrogen levels. Chemical examination disclosed that 50% of the zein protein fed daily was to be found undigested in the stools. The poor digestibility of this commercial zein was attributed to processing since many animal experiments indicate that this is not a characteristic property of native zein (Rathmann 1954).

In a series of studies sponsored by the Bureau of Biological Research of Rutgers University in which 6 reference proteins were evaluated by different methods of assay, paradoxical findings were obtained in regard to the nutritional value of *wheat gluten*. Mitchell (1950) reported that by the nitrogen balance method wheat gluten had a biological value of 40 as compared to 97 for egg albumin in the immature rat. Frost (1950), by the rat depletion method found a value of 9 for wheat gluten as compared to 38 for egg albumin. Chow (1950) on the other hand by repletion tests in dogs obtained a value of 135 for total circulating proteins with wheat gluten as compared to 117 with egg albumin after 2 weeks and 135 and 132 respectively after 4 weeks of repletion with these proteins. It is at once clear from the findings of this group of investigators that there may exist a lack of correlation between the nitrogen balance maintenance and plasma protein regenerative properties of protein foods. Albanese (1953b) studied the protein value of lysine supplemented wheat gluten for human infants. He found that with a wheat gluten milk containing no added lysine nitrogen retention fell precipitately and leveled off at about 50 mg per kilogram per day. Lysine supplementation was begun at the ninth week of the study and increased stepwise over 5 weeks at the rate of 1% of lysine per week. Nitrogen storage began to increase with supplementation and by the end of the third week the lysine reinforced wheat gluten milk was yielding nitrogen retention levels similar to those found with the evaporated milk formula.

In general mixtures of vegetable proteins as they are customarily employed are claimed to support fairly normal growth. Dean (1949) found that in infants under 3 months of age a normal growth occurred when 60-80% of the milk was replaced by a malted mixture of barley

wheat, maize flour, and soya meal. Diet surveys conducted in Mexico, Jamaica, Central America, Chile, Ceylon and several parts of Africa show that the diets consumed by children in all of these places have a striking similarity in the foodstuffs actually eaten. protein foods of animal origin are practically never ingested. The most prevalent combinations are beans and corn, rice and corn and wheat and corn. Growth and development of children in these areas are subnormal by American standards. Recent studies by Gomez *et al* (1958), Senecal (1958) and Behar and co workers (1958) on the nutritive merits of these mixtures will be discussed later.

### 6 Protein Digests

Possible therapeutic usefulness of protein hydrolyzates in the management of malnutrition and allergenic states of infancy prompted the author and his collaborators to undertake a systematic study of the biological value of these products in the infant. Albanese *et al* (1947b) observed that the nitrogen retention and weight gain of 3 normal infants maintained on a synthetic diet in which a tryptophan and cystine reinforced acid digest of casein constituted the principal source of nitrogen were respectively about 30 and 50% lower than those obtained when the same subjects were fed synthetic diets at the same fluid, caloric distribution and nitrogen levels in which enzymatic digests of casein or lactalbumin supplied the principal nitrogen component. The biological value of these latter two products, as indicated by the criteria of weight accretion and nitrogen storage appears for short periods to be almost equal to that of diluted evaporated milk formula and to that found by previous investigators employing various modifications of cows milk.

In a subsequent investigation Albanese *et al* (1948b) found that children, fed synthetic formulas with enzymatic digest of beef muscle as the principal source of nitrogen, showed a nitrogen retention and weight gain somewhat greater than routine evaporated milk formulae of equal nitrogen, caloric and fluid content. Later Albanese and co workers (1951) studied male infants on synthetic milk diets with the principal source of nitrogen as enzymatic digest of bovine plasma, as compared with infants fed routine evaporated milk formulas of equal caloric and nitrogen levels. They found that the bovine plasma digest group showed higher nitrogen retention and weight gain, and noted that the addition of isoleucine and methionine was without benefit. The authors then felt that nitrogen derived from a source which includes some peptides might be of some increased nutritional value when compared with a mixture of completely free amino acids. On the basis of these and other studies Cox and his associates (1947) reported that nitrogen retention in humans

when all the nitrogen was furnished by casein hydrolyzate showed no increase when supplemented by methionine (contrary to the experience with rats). They suggested that the increased amount of hair in the rat may be responsible for an increased need of the sulfur containing amino acids.

Clinical experience has indicated that protein digests are safe and therapeutically useful in sick or convalescent children. Hartmann and co-workers (1944) found that parenterally administered enzymatic digests of casein were nutritionally helpful to infants suffering from a wide variety of diseases which prevented adequate intake of food. Subsequently Langlois (1947) and Young *et al.* (1949) reported the use of protein digests in sick infants and found them clinically beneficial and without untoward effects.

In summary it appears that the findings of the majority of observers agree with the allowances recommended by the Food and Nutrition Board of the National Research Council (1958) for an intake of protein of 35 gm per kilogram per day in a diet containing 120 calories per kilogram per day during the first year of life. A search of the literature reveals a lack of quantitative observations on sufficiently large groups of breast fed infants as to the average quantity and average composition of the breast milk consumed by an infant during the whole 24 hours over a span of months under natural conditions of breast feeding. This circumstance and the numerous observations that growth and development of breast fed infants is greatly improved by supplements of meat or vegetable proteins preclude the soundness of claims for the use of estimated intakes of breast milk (16–22 gm or less of protein per kilogram per day) as a measure of the protein needs for the optimal growth and development of infants.

## C REQUIREMENTS FOR SPECIFIC AMINO ACIDS

### 1 Procedures

The need of precise knowledge of nutritional requirements is an obvious one. Such knowledge is essential for the recognition and intelligent repair of defective dietary situations. It is of particular importance in disturbances of the digestive tract where there are limitations in the amount of food that can be given. Because the production of an experimental diet deficient in a single amino acid which can be supplemented by known amounts of the amino acid in question involves particular difficulties information in regard to human requirements for amino acids has been slow in coming. In approaching this problem several types of experimental diets have been employed.

*a The use of natural proteins deficient in some particular amino acid* This method has limited applicability since such proteins occur primarily in vegetable and cereal foods. However, these foods comprise the principal source of protein for the major populations of the world. Consequently, determinations of the quantity of the test amino acid required under such conditions are of considerable practical as well as fundamental value.

*b The calculation of the intake of amino acids on known diets compatible with health* This method gives an approximate figure. One can say only that the requirement must be less than the intake so calculated. However, if criteria of good nutrition can be improved by the addition of one or more amino acids, then an accurate measure of the test amino acid need can be made under conditions of normal and natural dietaries.

*c The use of chemically degraded proteins or protein hydrolyzates* A number of procedures are known which destroy one or more specific amino acids. Diets constructed from such degraded preparations have the advantage that the amino acids are for the most part present as natural isomers and that the unessential, though physiologically important amino acids are present, thus avoiding the necessity of their biosynthesis. The disadvantage is that the nature and possible effects of the degraded fragments are unknown and can be readily overcome by chromatographic and chemical analyses.

*d The use of synthetic amino acid mixtures* Such diets have the advantage that all the components are known, but the disadvantage is that some of the amino acids have had to be supplied as racemic forms. The extent to which the unnatural isomers were utilized and the unknown effects on nonutilizable amino acids introduced uncertainties in these experiments. Furthermore, the fact that most of the nonessential amino acids are for the most part omitted, requiring their synthesis from essential amino acids or nonspecific nitrogen compounds, detracts somewhat from the usefulness of the data.

*e The use of a diet in which the protein moiety is provided by a mixture of pure amino acids—both nonessentials and essentials—all in the form of the natural optical isomers* In recent years the L forms of all the common dietary amino acids have become available. This approach in common with methods *c* and *d* suffers from the fact that all the dietary nitrogen is provided as free amino acids—an unphysiological form. Known differences in renal threshold of the free amino acids (Wright 1948) indicate that the pattern of amino acids available to the body for tissue synthesis will be quite different from the mixture fed.

## 2 Comparison of Available Data

Examination of the literature indicates that on a body weight basis, the nitrogen needs of the infant as provided by the usual cows milk formula are four to five times higher than those of the adult (Albanese *et al* 1947a). The available data however, do not reveal whether these higher protein requirements of the infant arise from a proportionate increased need for all the amino acids or from a limiting effect created by greater demands of the growing organism for one or several of the amino acids. The lack of and the need for, this information which is of obvious importance to practical infant dietetics and the physiology of growth in general which includes proliferation of benign and malignant neoplasms prompted the author and his collaborators to undertake some years ago a continuing program for the study of the amino acid needs of the infant and the growing child. In all of these studies the requirement of an essential amino acid was estimated from the additional amount needed to restore to physiological levels the nitrogen retention and rate of body weight gain of subjects previously maintained, for varying periods on diets poor in the test amino acid. The diets contained approximately 100 calories per 100 gm; the percentage caloric distribution being as follows: protein 14, fat 36 and carbohydrate, 50. The protein moiety consisted in all instances of proteins or protein preparations lacking in one of the essential amino acids. The observations were all made on normal male children who were given the diets in five feedings daily at the rate of approximately 100 calories and 0.56 gm nitrogen per kilogram of body weight and 50 mg of ascorbic acid together with 15 drops of *Oleum Percomorphum* daily. The diet periods were of 7 days duration and consecutive. The infants were weighed daily during the course of the experiment. The data on nitrogen retention were calculated from the results of nitrogen determinations of the 24 hour urine collections, analysis of the pooled feces for each period and from computations of the daily nitrogen intake based on food consumption records and the known nitrogen content of the diets. Measurements of the hemoglobin, total plasma proteins, albumin, globulin and NPN were done during each period interval.

On the basis of changes in rate of weight gain and nitrogen retention induced by fractional supplementation of the tryptophan deficient diet it was estimated that the infant requires approximately 30 mg of L-tryptophan per kilogram of body weight per day (Albanese *et al* 1947a). This value is approximately five times the adult tryptophan requirement previously reported by Holt and associates (1944) and suggests that the high milk protein need of the infant is predicated in part by the tryptophan requirement.



In experiments of similar design, using acid hydrolyzed beef hemoglobin as the isoleucine poor moiety of the diet, it was found that the normal infant requires approximately 90 mg of L isoleucine per kilogram per day which is approximately one third the quantity provided by the usual evaporated milk formulas (Albanese *et al* 1948a)

The cystine and methionine requirements of infants were determined by using a diet deficient in these 2 amino acids as the principal source of nitrogen, which was supplemented with varying proportions of L cystine and L methionine. Thus it was found that infants who received no cystine supplements required 85 mg of L methionine for the attainment of normal nitrogen retention and body weight gains. The infants who received diets containing 1% L cystine required only 65 mg of L methionine for restoration of the growth criteria to physiological levels (Albanese *et al* 1949a). On the basis of results obtained with a fractionally L lysine supplemented wheat gluten diet, it appears that the infant requires some 170-200 mg of L lysine per kilogram per day for the establishment of normal growth vectors (Albanese, 1949) (See Fig 3 Chapter 11 this volume)

Observations with the saturation point test wherein the amino acid requirements are measured in subjects who are maintained in normal nitrogen metabolism by natural foods fed *ad libitum* rather than in depleted subjects we have found that 3 months old children need 205 mg per kilogram of L phenylalanine per day for maintenance of normal blood phenylalanine levels 6 to 8 months old children require 170 mg per kilogram of L phenylalanine per day (Albanese *et al*, 1950)

From data available on the biological value of various proteins and protein products in the infant and analytical data on their composition it is possible to make some valid calculations of the minimal quantities of each amino acid needed for good nutrition when all of the amino acids are provided in the diet. Results of these calculations are collected in Table VII (Albanese 1951). The minimal quantities given in the last column should be considered as useful approximations pending more exact determinations. Autret (1955) reported that analysis of foods showed that these amino acid requirements for a 6 kg infant are met by 100 gm of whole wheat plus 35 gm skimmed milk powder whole wheat plus 50 gm pulses whole wheat plus 15 gm fish meal.

Snyderman (1958) has recently reviewed some preliminary observations on the amino acid needs of infants. Her group employed mixtures of crystalline amino acids purported to simulate the composition of breast milk proteins. Lack of some experimental details precludes a critical evaluation of these important studies. In general however it appears that the minimal amino acid needs found in this manner are about one

TABLE VII  
INFANT AMINO ACID REQUIREMENTS FOR NORMAL NITROGEN RETENTION AND BODY WEIGHT GAINS<sup>a</sup>  
Mg/kg/day

Amino acids	Cow's milk products			Beef products			Wheat gluten	Minimal quantity
	Evapo- rated milk	Casein (Amigen)	Lactal albumin (Lidamin)	Beef globulin	Beef muscle protein	Beef plasma protein		
Arginine	127	160	140	141	206	245	126	126 b
Histidine	63	93	81	277	134	112	70	63 b
Lysine	200	264	304	304	320	342	80 (170)	170 a
Tryptophan	47	70	87	59	93	49	30	30 a
Phenylalanine	177	199	213	250	169	182	246	169 c
Methionine	99	130	98	95	117	88	201	80 a
Threonine	151	157	206	257	234	245	87	87 b
Leucine	490	450	556	610	425	356	889	425 b
Isoleucine	167	251	227	36 (90)	102	126	195	90 a
Valine	171	263	161	298	227	266	182	161 b
Tyrosine	172	251	203	160	126	49	234	126 b
Cysteine	27	18	109	18	38	19	46	18 b
Serine	160	227	172	182	201	—	—	160 b
Glutamic acid	680	800	470	200	650	588	860	200 b
Aspartic acid	166	222	340	283	380	370	350	166 b
Glycine	11	17	0	—	210	—	315	0 b
Alanine	75	106	98	280	140	—	175	75 b
Proline	250	286	140	185	210	—	350	140 b
Hydroxyproline	—	70	—	—	—	—	—	70 b

<sup>a</sup> These values were obtained by three procedures (a) determinations from the amount of amino acid needed to restore nitrogen retention and body weight gain to physiological levels of subjects previously maintained for short periods on diets poor in the test amino acid (b) estimation from minimal amino acid content of proteins and protein products found to have high biological value in terms of nitrogen retention and body weight in infants and (c) calculations from saturation point of a test amino acid which is added stepwise to the diet of subjects maintained in normal nitrogen metabolism by natural foods fed *ad libitum*. Values in parentheses indicate concentration of amino acid after supplementation.

half those reported by Albanese (Table VII). It is the contention of this group that their minimal requirement values have nutritional validity because they approximate the amino acid intake of breast fed infants. The soundness of this contention must remain in serious doubt until such time as the previously discussed vagaries of the quantity and quality of protein ingested by breast fed infants can be adequately resolved.

*a Lysine* Because of its practical importance it seems worth while here to examine some probable procedural causes for the differences in lysine needs of infants as found by Albanese and Snyderman. In the studies of Albanese chemically defined wheat gluten, a lysine poor protein, served as the basic source of nitrogen in a diet of specified caloric distribution and vitamin and mineral content. These facts are not completely known regarding the diets employed by the present Bellevue group. The lysine poor (unsupplemented wheat gluten) periods in the authors studies were from 6 to 10 weeks duration as contrasted to on 7 days of lysine deficiency in the reported Snyderman experiment (Fig. 6). In these investigations the lysine requirement is defined as the amount of the amino acid (89 mg per kilogram per day) which induces the first increase in nitrogen retention—not necessarily the optimal value. In common with all studies of this design one is confronted here with the problem that a depleted organism utilizes a reincorporated nutrient more efficiently than does a well nourished organism. This biological effect of the law of the minimum can be overcome in part by prolonging the supplement periods until nitrogen retention is stabilized, and to determine the effect of positive increments in supplement before attempting decrements. Unless this is done one measures minimal requirement needs of the test nutrient in depleted infants rather than the nutrient requirement of healthy infants for normal growth and development. These matters were carefully considered in the interpretation of the authors studies, but apparently not in those of Snyderman and her associates. Several evidences for the law of the minimum effects occur in the Snyderman data. Thus after the lysine free regimen (Period 3), the addition of 88.4 mg of lysine to the diet (Period 4) resulted in a nitrogen retention of approximately 280 mg per kilogram per day. In Period 6 89 mg of lysine HCl induced a nitrogen retention of only 120–130 mg per kilogram per day. It is also to be noted that the rate of body weight gain at the 89 mg of lysine HCl per kilogram per day level of the infant shown in Period 4 (Fig. 6) was some 50% less than that attained on the amino acid mixture providing 278 mg of lysine HCl per kilogram per day. Finally, at the completion of the study this infant should have weighed approximately 80 kg as contrasted to the observed 73 kg (Naimark 1957).

## D AMINO ACID PATTERNS AND NITROGEN NEEDS FOR GROWTH

On the basis of available evidence (Berch *et al*, 1943 Mitchell 1950) proposed that amino acid analyses of mammalian carcasses might prove to be an effective method for estimating the requirements of some if not all of the essential amino acids for growth. From data on the amino acid requirements of infants found under uniform and controlled dietary con

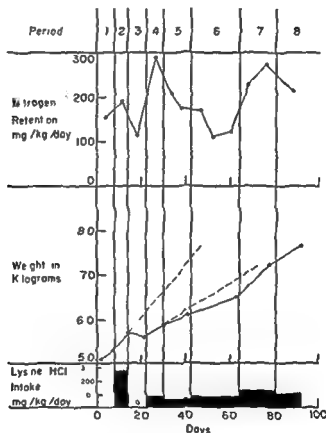


FIG 6 Lysine intake, body weight gain, and nitrogen retention of 15-month old infant. Adapted from Snyderman (1957)

ditions (Albanese 1950) a growth pattern of amino acid requirements was obtained by assigning value of unity to the tryptophan need (Table VIII). A similar calculation of the amino acid content of mammalian tissues recorded by Mitchell showed that there exists good agreement of growth needs and tissue amino acid patterns. This agreement is particularly good for the lysine/tryptophan (L/T) and methionine/tryptophan (M/T) ratios of muscle proteins which constitute approximately 75% of the infant body proteins.

In the light of these data and the lysine and tryptophan content of

foods commonly employed in the feeding of infants (Albanese *et al* 1955a), it was felt worthwhile (a) to re-examine the nutritional value in infants of various dietary proteins in terms of their lysine/tryptophan (L/T) ratios, and (b) to explore the nutritional effects of graded lysine supplements to dietaries having L/T ratios below 63 (that of muscle proteins).

TABLE VIII  
PATTERN OF AMINO ACID NEEDS OF INFANTS COMPARED TO THE COMPOSITION OF MAMMALIAN TISSUES<sup>a</sup>

Amino acids	Mg/kg/day	Male infant needs <sup>b</sup> Growth pattern	Mammalian tissue patterns <sup>c</sup>		
			Muscle proteins	Viscera	Plasma proteins
Tryptophan	30	10	10	10	10
Lysine	180-210	60-70	63	53	62
Methionine	85	28	25	20	11
Isoleucine	90	30	45	33	20

<sup>a</sup> Growth and tissue pattern figures were obtained by assigning a value of unity to tryptophan (Albanese 1956)

<sup>b</sup> Albanese (1950)

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The relative content of the critical amino acids of the evaporated milk and test formulas, as compared to the measured amino acid needs of infants (2-9 months) is shown in Table IX. It will be noted that the

TABLE IX  
AMINO ACID PATTERN OF INFANT NEEDS COMPARED TO SOME PROTEIN PRODUCTS<sup>a</sup>

Amino acids	Infant needs	Evaporated milk		Lactalbumin (Eda min)	Beef muscle digest	Bovine plasma (Trava min)	Wheat gluten
		Casein (Amgen)					
Tryptophan	10	10	10	10	10	10	10
Lysine	60-70	43	39	35	59	69	20
Methionine	28	21	20	11	26	17	30
Isoleucine	30	36	38	26	36	27	33

<sup>a</sup> These relative values were calculated from the best amino acid analytical data available for the products employed. In most instances these data were found to check with triplicate analyses in our laboratory by methods published by the author and his associates. Different ratios will obtain when other analytical data are employed but the differences will be relative rather than absolute (Albanese 1956).

recorded L/T ratio for evaporated milk is slightly lower than that reported for whole cow's milk (Macy *et al* 1953). This difference may be due in part to the destruction of lysine during processing of the milk (Mauron *et al* 1955). Since the proteins of all products tested con

tained approximately 1% tryptophan and were fed at the uniform level of 35 gm of protein per kilogram per day the diets in these studies all provided approximately 35 mg of tryptophan per kilogram per day. This is an amount of tryptophan which we have found to cover the measured infant need of 30 mg per kilogram per day (Albanese *et al.*, 1947a).

In order to arrive at a relative utilization value of the test protein products the following formula was applied to our results:

$$P = BW \times N/1000$$

where  $P$  = protein utilization  $BW$  = body weight change in grams per day and  $N$  = nitrogen retention in milligrams per kilogram per day.

The coefficient of utilization of the test products  $P_r$ , is then expressed as the numerical value of the ratio  $P$  test protein formula/ $P$  evaporated milk formula or  $P_r = P_{tp}/P_{em}$ . Expression of the bio assay results in this manner has several advantages. First it equalizes disparities between body weight changes and nitrogen retention values which often arise in infants from transpositions of body fluid compartments. Secondly it directly relates increments in nitrogenous tissue to qualitative amino acid differences of the test nitrogenous moiety of the diets. And lastly it provides a simple numerical comparison of the test substance with a standard infant food—evaporated milk.

An example of such calculations for a male infant W. M. weighing 3327 gm at 15 months of age follows. Consumption of a standard evaporated milk formula at the average level of 311 gm nitrogen per day for 2 weeks resulted in an average body weight change of 23 gm per day and a nitrogen retention of 138 mg per kilogram per day. Thus  $P_m = 23 \times 138/1000$  or 3.17. During the subsequent 2 weeks this infant consumed an isocaloric bovine plasma digest formula at the average rate of 312 gm nitrogen per day. This dietary resulted in an average body weight change of 40 gm per day and a nitrogen retention of 180 mg per kilogram per day. Thus  $P_{tp} = 40 \times 180/1000$  or 7.20. Hence the coefficient of utilization ( $P_r$ ) of the bovine plasma digest formula in this single assay is  $7.20/3.17$  or 2.3. The average  $P_r$  of ten of such bio assays in infants 15–90 months of age was 1.82.

The details and results of this comparison are collected in Table X. These data clearly show that bovine plasma and beef muscle digest are more effectively utilized for growth by the infant than are cow's milk proteins or their products. Reference to the data contained in Tables VIII and IX leads to the impression that greater biological availability of the bovine products may be associated with the approaching coincidence of their lysine and tryptophan content with (a) the lysine/

tryptophan ratio needs of the infant, and (b) the lysine/tryptophan ratio of the predominant infant tissues. It is also clear from our studies that the possible correlation of the L/T ratios of protein substances and their utilization in infants prevails only for products which have over all amino acid patterns not too greatly different from tissue proteins, and which provide 30 mg or more of tryptophan per 35 gm of protein, or approximately 10% of tryptophan. A substance like gelatin, which has an L/T of infinity, by virtue of its lack of tryptophan could not be expected to have exceptional nutritional values unless its primary amino acid deficiencies and imbalances are corrected. As a matter of fact,

TABLE X

THE LYSINE/TRYPHTOPHAN (L/T) RATIO OF VARIOUS PROTEIN PRODUCTS AND THEIR UTILIZATION IN INFANTS\*

Protein source	L/T of product	Subjects		Average coefficient of protein utilization (Pr)
		No	Age (mo)	
Lactalbumin digest (Edamin)	3.5	11	5.0-11	0.89
Casein digest (Amigen)	3.9	6	5.0-11	0.95
Evaporated milk	4.3	38	1.0-27	1.00
Beef muscle digest	5.9	5	2.5-8.0	1.43
Bovine plasma digest (Travamin)	6.9	10	1.5-9.0	1.82
Wheat gluten	2.1	6	3.0-8.0	0.52
Wheat gluten plus 2.0-3.0% L lysine	5.0-6.0	6	3.0-8.0	1.05
Evap. milk plus 2.0-4.0% L lysine	6.0-8.0	8	1.0-27	1.75
Amigen plus 2.0-4.0% L lysine	6.0-7.0	3	6.0-9.0	1.65

\* All diets were fed at the rate of 3.5 gm of protein ( $N \times 6.25$ ) and 100 calories per kg per day (Albanese 1956)

unpublished studies in our laboratory have shown that the amino acid imbalance of gelatin is such that its nutritive value cannot be greatly improved by supplementation of its deficiencies by crystalline amino acids.

The following observations also made during the course of these studies seem particularly worthy of note in the development of our thesis.

Supplementation of the casein digest (Amigen) with 1% of DL methionine was found not to improve the utilization of this product (Cox *et al*, 1947). In view of the carcass method concepts this result could have been anticipated since the methionine/tryptophan (M/T) ratio of Amigen closely approximates the average for mammalian tissues (Table IX). Also in retrospect, our failure to improve the utilization of the bovine plasma digest (Albanese *et al*, 1951) by the combined addi-

tion of 1% DL isoleucine and 0.6% DL methionine can also be ascribed to the fact that the isoleucine/tryptophan and methionine/tryptophan ratios of the commercial product (Trivamin) are in approximate agreement with those prevailing in mammalian tissues (Tables VIII and IX)

### 1 *Effects of Lysine Supplementation on Protein Utilization*

A careful consideration of the foregoing and in particular the data contained in Table X eventually led to the thought that infant utilization of milk proteins could be improved by additions of lysine. Calculations indicated that L lysine supplements of 50-100 mg per kilogram per day might prove effective with infants receiving 35-40 gm of evaporated milk proteins per kilogram per day. Lysine supplements of this order of magnitude would increase the L/T ratio of the evaporated milk formulas from 4.3 to 6.0-8.0.

Preliminary studies on 15 infants showed that supplementation of the diet of infants with poor appetites with 100 mg per kilogram per day of L lysine was associated in most instances with a marked increase in daily nitrogen retention and body weight as well as significant increases in blood protein levels (Albanese *et al.* 1955a).

Examination of these and subsequently published data (a total of 27 infants) indicates that improved nutrition in terms of body weight change (a commonly accepted criterion of nutritional state) occurred when L/T ratios of 6.0-7.0 were maintained by lysine additions to diets offered at a minimum of 100 calories and 35 gm of protein per kilogram per day. Increased milk protein utilization as previously defined under these conditions is shown in Table X. It will be noted that lysine reinforced milk proteins attain a degree of utilization comparable to that of bovine muscle and plasma digests. Our data further indicate that the nutritive value of a casein digest (Amigen) a product often employed in the feeding of milk allergic infants may also be improved by additions of lysine (Table X).

Vargas Rubiano and associates (1958) and Vignec (1958) have reported studies which provide confirmatory evidence of the efficacy of lysine fortification of evaporated milk dietary of nutritionally substandard infants.

The sum total of these observations clearly suggests that the carcass method for the estimation of the amino acid needs has some applications in human nutrition. One of the most promising of these applications may be based on the apparent relationship of the L/T ratios of proteins and their utilization. Accordingly, it was of some interest to find on algebraic analysis that the utilization coefficient of dietary proteins ( $P_r$ ) as pre-



viously defined, is linearly related to the lysine/tryptophan (L/T) ratio of six protein products by the equation,

$$P_r = 0.28 (L/T) - 0.15$$

A graphic representation of the fit of the experimental data to the line of this equation is shown in Fig 7. As previously noted these considerations apply only to proteins which contain a minimum of 1.0% tryptophan, and present an amino acid contour which approximates that of the tissue proteins. Further support for this relationship is derived

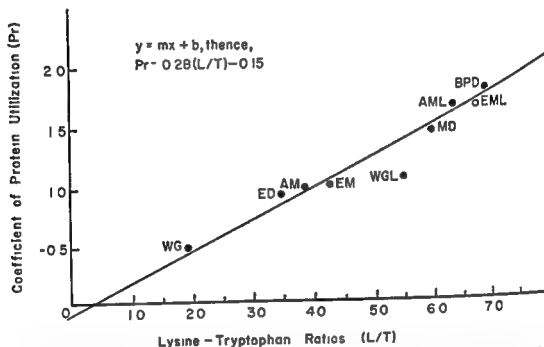


FIG 7. Algebraic relationship of the L/T ratios of some protein products and their coefficient of utilization in infants. The products tested are designated as follows: WG wheat gluten, ED Edamun, AM Amigen, EM evaporated milk, WGL wheat gluten with lysine, MD meat digest, AML Amigen with lysine, EML evaporated milk with lysine, BPD bovine plasma digest (Travamin) (Albanese 1956).

from the findings that the increase in the value of  $P_r$  for L-lysine reinforced evaporated milk or casein digest formulas is in good accord with that expected from the augmented L/T values of 65-70. Calculations show that the nutritional value of lactalbumin, which has a low L/T value by virtue of its high tryptophan content, might be greatly extended by small additions of lysine.

Modification of the L/T ratio of a wheat gluten diet from 20 to 55 by additions of lysine was found to improve its nutritional value (Table X) to that of the milk protein diets (L/T 35-43) (Albanese *et al.* 1949b, Hoffman and McNeil 1949). However, this improvement fell short of

that expected from the equation (Fig 6). At first sight, this anomaly seemed to point to the existence of a limiting level of some other essential amino acid or acids in wheat gluten. Reference to Table IX shows that methionine and isoleucine are probably not implicated in this nutritional shortcoming of lysine reinforced wheat gluten. However, consideration of the investigations of Williams and co-workers (1954) which lend considerable support to the carcass theory of amino acid requirements for growth in rats also suggests that the observed nutritional limitation of wheat gluten probably arises from an amino acid imbalance created by the presence of excessive amounts of glutamic acid (Blush 1945). The importance of these possible relationships in attempts to improve the nutrition of populations of underdeveloped world areas, whose diets are comprised largely of wheat and other cereal products, is obvious.

These evidences support the conclusion that the carcass analysis procedure proposed by Mitchell may be a valid method for evaluating the human growth needs for some "essential" amino acids. In particular, the available evidence indicates that the utilization of dietary proteins increases as their lysine and tryptophan content approaches that of muscle tissues. This concept gains further validity from the fact that the nutritional value of some protein products with low lysine/tryptophan values can be enhanced by small additions of lysine. In milk protein products this increased utilization approaches that of bovine muscle and plasma digests and appears to be a linear function of the augmented lysine/tryptophan values. Lysine supplementation of wheat gluten increases its nutritive value to that of milk protein products.

### E. CALORIES AND PROTEIN NEEDS

The incorporation of a series of individual amino acids into a protein molecule requires energy and this energy is derived largely from carbohydrates and fats. The actual transference of the energy to the synthesizing mechanisms is presumably accomplished by the intermediation of energy rich phosphite bonds. It is probable therefore that tissue protein synthesis can proceed efficiently only when there is a readily available source of energy derived either from dietary carbohydrate or fat or from tissue stores particularly of fat. But because a less than optimal caloric intake is often associated with an impaired utilization of amino acids it can be reasonably assumed that for amino acid utilization in general an optimal daily caloric intake is also necessary.

Also it has long been known that nitrogen balance can be achieved without carbon equilibrium (Lusk 1928). The carbon and nitrogen contents of the urine give significant information on the physiological fuel

value of protein, carbohydrate, and fat. Normally, every gram of urinary nitrogen is accompanied by the elimination of 0.6-0.9 gm of carbon. Shifts in the urinary C/N ratio have been found useful in the diagnosis of abnormal metabolic states (Macy, 1942).

### *1 Effect of Carbohydrate Calories*

In 1856, Hoppe reported that carbohydrate ingestion lowered the nitrogen excretion of dogs. This finding was later corroborated by Lusk (1928) by a reverse experiment on himself which demonstrated that the withdrawal of 350 gm of carbohydrate from the diet at two levels of nitrogen intake (20.55 and 9.23 gm per day) increased his urinary nitrogen output by 4 gm above the usual values in both instances. Thus protein sparing action of the carbohydrates was also found to occur in the fasting dog (Rubner, 1883) and man (Landergren 1903). These observations bear evidence that the carbohydrates can spare the nitrogen needs of the mammalian organism at nearly all levels of protein intake. The practical implications of this phenomenon were demonstrated by the experiments of Keller (1899), Orgler (1908), and Rosenstern (1918), which demonstrated that the nitrogen retention of infants from a given amount of protein could be greatly augmented by increasing the carbohydrate content of the diet.

### *2 Nitrogen Sparing Value of Various Carbohydrates*

Teleological reasoning has led many investigators to the conclusion that because lactose is the naturally occurring sugar of the milk of all mammals this sugar should possess nutritive characteristics for nurslings which are lacking in other carbohydrates. These qualities have been associated by conjecture with the need for galactose in the formation of nerve tissues or the rate of absorption of galactose which is more rapid than that of glucose or the influence of galactose upon calcium metabolism. Speculations on unique effects of lactose on the nitrogen metabolism have also been entertained. Measurements by the author and his associates of nitrogen retention of normal infants fed cow's milk formulas supplemented isocalorically with lactose and Dextrimaltose do not support these speculations (Table XI). On the other hand no untoward effects such as diarrhea or poor assimilation were noted in these studies. The fear that lactose supplementation of infant diets may induce cataract formation is unfounded in fact since (a) the incidence of galactosemia is no greater in breast fed children than in those artificially fed where other sugars are commonly used and (b) the cataractogenic effects of lactose in rats are noted only with diets containing 30% or more of the carbohydrate (Mitchell and Dodge 1935, Bruck and Rapaport 1945, Riggs and Beaty 1947).

In recent years the nutritional advantages of sucrose and dextrose have been studied intensively in this laboratory. It may be expected because of the lability of the fructose molecule, that sucrose would bear biochemical qualities not available with diets containing dextrose alone. Early experiments reported by Albanese and associates (1947c) on the effect of carbohydrate feeding on the output of urinary amino acids disclosed that single feedings of 25 gm of sucrose had a greater sparing effect on the nitrogen and tryptophan output of young male and female adults than did 25 gm of glucose.

TABLE VI

EFFECT OF DEXTRIMALTOSE AND LACTOSE ON NITROGEN RETENTION AND FECAL NITROGEN OF NORMAL MALE INFANTS\*

Subjects	Diets	DMM <sup>b</sup>	DMM	DMM	LM	LM
DS 3 mo	N retention gm/day <sup>c</sup>	0.89	0.79	0.87	0.68	0.89
40 kg	Fecal N gm/day	0.50	0.39	0.35	0.61	0.34
RJ 4 mo	N retention gm/day	0.82	0.52	0.69	0.75	0.38
35 kg	Fecal N gm/day	0.44	0.40	0.49	0.44	0.40
WM 8 mo	N retention gm/day	1.32	1.00	0.84	1.14	0.71
73 kg	Fecal N gm/day	0.60	0.56	0.64	0.49	0.37
FS 11 mo	N retention gm/day	1.13	1.09	0.95	0.86	0.95
33 kg	Fecal N gm/day	0.46	0.32	0.34	0.32	0.29

\* Albanese (1950)

<sup>b</sup> Diet composition: Evaporated milk 1100 ml, Dextrimaltose No. 2 154 gm in DMM or lactose USP 150 gm in LM, water to 2640 ml. Both formulas were given at the rate of 3.5 gm of protein and 110 cal per kilogram per day.

<sup>c</sup> All results given as averages of 5 day periods.

Subsequent investigations (Albanese *et al.* 1955b) revealed that diets high in fructose induced a greater nitrogen retention in children than diets high in dextrose or galactose. The magnitude of this difference is shown graphically in Fig. 8. It will be observed that isonitrogenous diets containing 71% of the total carbohydrates as fructose induced nitrogen retentions of 30–50 mg per kilogram per day greater than those high in glucose or galactose.

These findings led the author and his co-workers to inquire further into the biochemical mechanism through which this end result was achieved. After some unsuccessful experiences they found that changes induced within one hour by different sugars in the blood amino nitrogen levels of fasting subjects could serve as a dynamic index of protein sparing activity of various carbohydrates (Albanese *et al.* 1955c). The data resulting from this investigation are summarized graphically in Fig. 9. Examination of the data discloses that within 60 minutes of ad-

value of protein, carbohydrate, and fat. Normally, every gram of urinary nitrogen is accompanied by the elimination of 0.6–0.9 gm of carbon. Shifts in the urinary C/N ratio have been found useful in the diagnosis of abnormal metabolic states (Macv, 1942).

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J 4 mo	N retention gm/day	0.82	0.52	0.69	0.75	0.38
5 kg	Fecal N gm/day	0.44	0.40	0.49	0.44	0.40
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3 kg	Fecal N gm/day	0.60	0.56	0.64	0.49	0.37
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In recent years the nutritional advantages of sucrose and dextrose have been studied intensively in this laboratory. It may be expected because of the lability of the fructose molecule, that sucrose would bear biochemical qualities not available with diets containing dextrose alone. Early experiments reported by Albanese and associates (1947c) on the effect of carbohydrate feeding on the output of urinary amino acids disclosed that single feedings of 25 gm of sucrose had a greater sparing effect on the nitrogen and tryptophan output of young male and female adults than did 25 gm of glucose.

TABLE VI  
EFFECT OF DEXTRIMALTOSE AND LACTOSE ON NITROGEN RETENTION AND FECAL NITROGEN OF NORMAL MALE INFANTS<sup>a</sup>

Subjects	Diets	DMM <sup>b</sup>	DMM	DMM	LM	LM
OS 3 mo	N retention gm/day <sup>c</sup>	0.89	0.79	0.87	0.68	0.89
0 kg	Fecal N gm/day	0.50	0.39	0.35	0.61	0.34
BJ 4 mo	N retention gm/day	0.82	0.52	0.69	0.75	0.38
5 kg	Fecal N gm/day	0.44	0.40	0.49	0.44	0.40
MM 8 mo	N retention gm/day	1.32	1.00	0.84	1.14	0.71
3 kg	Fecal N gm/day	0.60	0.56	0.64	0.49	0.37
TS 2 mo	N retention gm/day	1.13	1.09	0.95	0.86	0.95
3 kg	Fecal N gm/day	0.46	0.32	0.34	0.32	0.29

<sup>a</sup> Albanese (1950)

<sup>b</sup> Diet composition: Evaporated milk 1100 ml, Dextrimaltose No. 2 154 gm in DMM or lactose USP 150 gm in LM water to 2640 ml. Both formulas were given at the rate of 3.5 gm of protein and 110 cal per kilogram per day.

<sup>c</sup> All results given as averages of 5 day periods.

Subsequent investigations (Albanese *et al.*, 1955b) revealed that diets high in fructose induced a greater nitrogen retention in children than diets high in dextrose or galactose. The magnitude of this difference is shown graphically in Fig. 8. It will be observed that isonitrogenous diets containing 71% of the total carbohydrates as fructose induced nitrogen retentions of 30-50 mg per kilogram per day greater than those high in glucose or galactose.

These findings led the author and his co-workers to inquire further into the biochemical mechanism through which this end result was achieved. After some unsuccessful experiences they found that changes induced within one hour by different sugars in the blood amino nitrogen levels of fasting subjects could serve as a dynamic index of protein sparing activity of various carbohydrates (Albanese *et al.* 1955c). The data resulting from this investigation are summarized graphically in Fig. 9. Examination of the data discloses that within 60 minutes of ad-



value of protein, carbohydrate and fat. Normally, every gram of urinary nitrogen is accompanied by the elimination of 0.6–0.9 gm of carbon. Shifts in the urinary C/N ratio have been found useful in the diagnosis of abnormal metabolic states (Macy, 1942).

### *1 Effect of Carbohydrate Calories*

In 1856, Hoppe reported that carbohydrate ingestion lowered the nitrogen excretion of dogs. This finding was later corroborated by Lusk (1928) by a reverse experiment on himself which demonstrated that the withdrawal of 350 gm of carbohydrate from the diet at two levels of nitrogen intake (20.55 and 9.23 gm per day) increased his urinary nitrogen output by 4 gm above the usual values in both instances. This protein sparing action of the carbohydrates was also found to occur in the fasting dog (Rubner, 1883) and man (Landergrén, 1903). These observations bear evidence that the carbohydrates can spare the nitrogen needs of the mammalian organism at nearly all levels of protein intake. The practical implications of this phenomenon were demonstrated by the experiments of Keller (1899), Orgler (1908), and Rosenstern (1918), which demonstrated that the nitrogen retention of infants from a given amount of protein could be greatly augmented by increasing the carbohydrate content of the diet.

### *2 Nitrogen Sparing Value of Various Carbohydrates*

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WM ■ mo	N retention gm/day	1.32	1.00	0.84	1.14	0.71
7.3 kg	Fecal N gm/day	0.60	0.56	0.64	0.49	0.37
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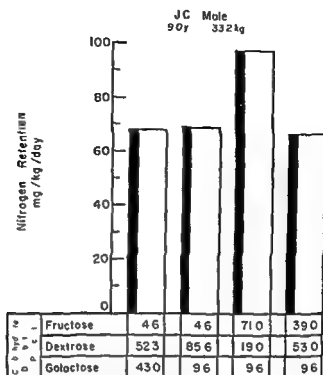


FIG 8 Effect of carbohydrate distribution on nitrogen retention of children. The percentile caloric distribution of the diets was protein 10, fat 35, and carbohydrates 55. Depending on age, the diets supplied 1.3-3.0 grams of protein per kilogram of body weight per day (Albanese *et al.* 1955b).

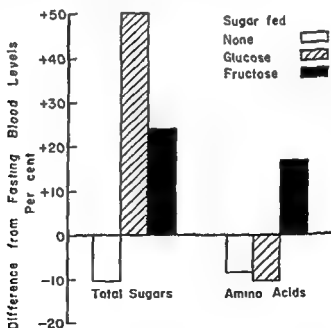


FIG 9 Effect of test doses of glucose and fructose (1 gm per kilogram of body weight) on total blood sugar and plasma amino acids (Albanese *et al.* 1955b).

ministration fructose causes an appreciable increase in blood amino nitrogen. In the same interval of time, glucose causes an increase in blood amino nitrogen no greater than that found on continued fasting of the subjects. Indeed the rise produced in blood amino nitrogen by the fructose was equivalent to that produced by the similar administration of 0.3 gm of milk protein per kilogram. Although at first glance these findings would seem to require a revision of the accepted mechanism for the mammalian metabolism of sugars, a more careful consideration reveals that there is no need for such a revision. In point of fact, the measurements simply suggest that fructose is a readier source of carbon radicals for the transamination cycle than dextrose. In the light of our nitrogen balance data this rate of difference may be interpreted to signify that fructose induces a greater salvaging of circulating non-protein metabolites than dextrose.

### F. MALIGNANT MALNUTRITION OF INFANTS (KWASHIORKOR)

Despite the great amount of effort which has been expended on the so called daily amino acid requirements it is difficult to interpret the results in their relationship to varying nutritional needs (Waterlow and Stephen 1957). In short the maintenance requirements of a healthy subject for remedying the consequences of ordinary metabolic tissue erosions cannot be applied to undernourished subjects in differing states of nutritional depletion such as occur in kwashiorkor. This syndrome variously known as Shibi Gachaki (Japan) Mehlhirschaden (Germany) Kwashiorkor (Ghana) Male di Farina (Italy) Syndrome Pluricarencial Infantil (Central America) and Protein Malnutrition (Gomez *et al* 1954), is the inheritance of children living chiefly in technically underdeveloped countries. A number of factors—social economic sanitary, and educational—contribute to its high incidence. The cumulative effect of the negative action of each of these factors bears on millions of individuals throughout the world and the prevention and treatment of the disease have thus become of late a world responsibility.

Some of the problems raised in the management of this nutritional syndrome were pointed up by the report of Cannon and associates (1953) on comparative studies demonstrating the varying daily utilization rates for each of the essential amino acids in healthy adult rats and in adult rats which had been subjected to marked protein depletion. Whereas it was evident that there was a definite maintenance requirement for each essential amino acid in the depleted animal this requirement was augmented in many instances from two to almost five times. It would seem from these experiments that under the conditions of depletion the tissue avidity for each essential amino acid is elevated more so for some than

for others. For example, the utilization rates for lysine and leucine were especially high in comparison with the others. The only explanation which these investigators could offer was that in the reconstitution of depleted tissues, where the greatest over all loss was of muscle mass, muscle tissue presumably required the greatest amounts, relatively, of the amino acids normally responsible for its characteristic chemical structure. In support of this idea is the evidence from amino acid analyses of normal beef muscle that the ratio of lysine and leucine to tryptophan is approximately 8 to 1 (Greenwood *et al.*, 1951).

### 1 Amino Acid Supplements

While the use of milk continues to be an ideal basis for therapy and prevention of kwashiorkor, properly constituted vegetable protein mixtures are almost equally effective for treatment and are the most promising practical means of prevention for many areas of the world. Amino acid supplementation of vegetable foods would seem to provide a practical solution in areas in which animal protein is in short supply. Indeed Gomez *et al.* (1958) encouraged by the good results obtained by the addition of limiting amino acids in the dietary of experimental animals, fed a diet of corn meal and beans fortified with lysine and tryptophan to four cases of infantile malnutrition. This supplementation was followed in all instances by an increased percentage of nitrogen absorption and retention. The beneficial effects of amino acid supplementation have been confirmed by Bressani and co-workers (1958) in hospitalized children afflicted with Kwashiorkor who were fed lysine and tryptophan fortified corn masa. Gomez *et al.* (1937) have also reported that graded lysine reinforcement (100, 250, 500 mg per day) of a cow's milk diet of 5 severely malnourished children resulted in improved retention of the intake in 2 and possibly 3 of the children.

Brock and associates (1955) have treated successfully 9 cases of kwashiorkor with an amino acid formula containing a known mixture of synthetic vitamins. Apparently, contrary to the claims of Gyorgy (1953) and Schwartz (1952), no unidentified dietary factors are required for the initiation of cure as defined by these investigators.

From these observations we may conclude that after maintenance requirements are finally established for each essential amino acid, the problem remains in the planning of a rehabilitation diet of allowing from two to five times the maintenance needs of each essential amino acid in order to furnish to the repleting tissues a rich assortment of these amino acids in proper proportions for their best utilization by the synthesizing mechanisms. Even more difficult, however, is the further problem of determining the associated needs of these mechanisms for energy,

vitamins salts and nonessential amino acids as essential interacting contributors to the tissue syntheses

## II PREADOLESCENCE

### A IOWA STATE STUDIES

Stearns and associates (1958) have recently reported on the thorough long range observations of her group on the protein requirements of children from 1 to 10 years of age. They noted that the period from 18 months to 3 years and perhaps to 4 years of age is the period wherein growth of the skeletal musculature is most rapid in relation to total body growth.

If the period of slow body growth is the period of most rapid growth of muscle tissue, it seems obvious that protein requirement will be high during this age range—probably as high as during infancy. It will be relatively high throughout the entire period wherein muscle growth is more rapid than total growth. The actual requirement at any age will vary with the rate of growth of muscle and with the child's efficiency of utilization of his intake.

In these investigations nitrogen was well absorbed at every age studied. The mean fecal nitrogen remained almost constant at about 11% of the intake throughout the entire age range reported here. The nitrogen absorbed but not returned is excreted in the urine. The various levels of protein intake were obtained largely through adjustment of the milk intake substituting nonprotein calories for milk as is commonly done in families.

The retention of nitrogen with increasing intake is shown on a per kilogram basis for children 1-4 years of age in Fig. 10. For comparison mean retention values are shown for infants given a similar range of intake. No significant difference in retention for any given intake can be noted among the children of these three ages. These data (Fig. 10) show that the retention of nitrogen by young children is somewhat smaller and less steady than that of the infants given comparable intakes. They also show that small children retain very little of a low nitrogen intake but the retention rises rather sharply between 400 and 500 mg nitrogen intake (25-3 gm of protein) per kilogram then apparently reaches a plateau only to show a second rise when the intake is increased above 650 mg (40 gm of protein). It is emphasized that these data refer to intakes attained voluntarily by children given dietary regimens consisting of common foods normally eaten by these age groups. The intake of protein foods was not forced nor was the diet "stuffed" by the addition of special proteins. Few of the children consumed amounts of protein over 45 gm per kilogram per day.

Nearly 80% of the data lie between intakes of 450 and 650 mg nitrogen (2.75-4 gm of protein) per kilogram of body weight. The mean retention for this entire group approximates 110 mg per kilogram, with a range of (approximately) 70-150 mg per kilogram. From the data an intake of 3 gm of protein per kilogram can be considered the minimum permitting a satisfactory retention of nitrogen for children 1 year of age. An intake over 3.5 gm per kilogram is no longer economical. An intake between 3.0 and 3.5 gm per kilogram or approximately 130 gm per pound, seems a safe allowance for children 1-4 years of age.

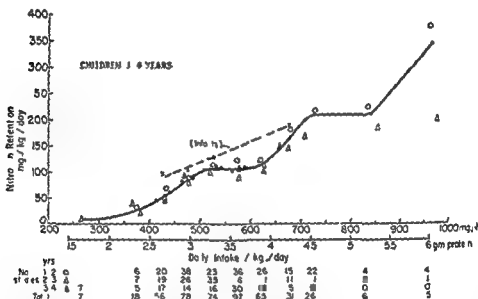


FIG 10 The retention of nitrogen per kilogram daily in relation to increasing per kilogram intake. Data for infants are shown as broken lines; those for children from 1 to 4 years are shown as solid lines. The symbols differ for each year of age. The mean retention values were obtained by averaging the retention data for all 3 age groups for each 25 mg of increase in intake (Stearns *et al.* 1958).

The curve of mean per kilogram nitrogen retention of 4 year olds given increasing amounts of protein differed significantly from that of children under that age.

Children 4 through 10 years of age showed no significant difference in mean quantity of retention for a given nitrogen intake. Therefore, the data for all children 4 through 10 years have been combined and the mean retention per kilogram daily is shown in relation to increasing per kilogram nitrogen intake in Fig 11. All showed some evidence of resisting loss of nitrogen at low intakes though the data are insufficient to be conclusive in either group. The retention of the older group leveled off at 46 mg of nitrogen per kilogram of body weight between intake of 300 and 400 mg of nitrogen per kilogram of body weight (about 2-2.5

gm of protein) With increasing intake of nitrogen, retention rose steadily, but at a slower rate than that observed in the young children. The second retention plateau at 100 mg of nitrogen per kilogram was achieved at intakes above 525 mg per kilogram (35 gm of protein per kilogram), or about the same intake level as was necessary to provide 100 mg retention for the young children.

When the retention curves for the two groups are compared, the chief differences observed are the increased retention of the older group as compared with the younger at intakes below 400 mg of nitrogen per kilogram of body weight and their relatively slower rate of rise as intake increased over 400 mg per kilogram. This made establishment of a precise requirement more difficult.

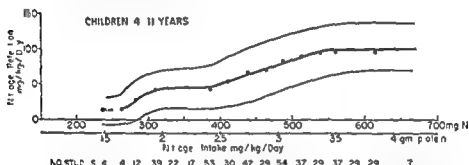


FIG. 11. Retention of nitrogen per kilogram daily in relation to increasing per kilogram intake data for children 1-11 years of age. The mean retention is determined by averaging all retention data for each 25 mg of increase in intake of nitrogen (Sterns *et al.* 1958).

The relative rate of growth of musculature for children 4-10 years old still is greater than rate of growth of the body as a whole although the difference in rates diminishes as the child approaches 9 years. On the other hand, annual rate of body growth is somewhat more rapid after 5 years so that total growth of musculature is still rapid. In addition, these are the kindergarten and early school years during which the common contagions are prevalent and the child develops his immunities. Bed rest alone lowers the retention of nitrogen and loss may be heavy during a febrile illness. Such loss of protein tissue is regained far more slowly than it is lost. Return to prior weight may not be synonymous with return to previous quantity of protein tissue. Again it appears wise to keep the requirement generous.

Each 100 mg rise in intake from 300 to 600 mg per kilogram includes about one third of the total data. The mean retention of those given 300-400 mg was 46 mg per kilogram. One half of the children retained less and one sixth retained less than 16 mg per kilogram. These



less efficient children in this age range may suffer nutritionally if the intake is limited to 2-25 gm per kilogram

An intake above 3.25 gm per kilogram per day appears uneconomical for this age range. An intake of 3 gm of protein per kilogram for the 4 to 7 year olds, decreasing gradually as the child grows older but keeping well above 2.5 gm per kilogram per day, will permit maintenance of growth of muscle and allow sufficient protein for recovery from such exigencies as febrile and other illnesses common to children. The children studied have shown no evidence that protein intake levels of 3 gm per kilogram or more have any untoward effect. There is no reasonable objection to maintenance of 3 gm of protein per kilogram per day for the active school child. It is insurance against deleterious influences, and permits him to endure the stress of rapid circumpuberal growth without any added stress of nutritional deficiency. As protein foods are carriers of so many of the other nutrients essential for good health, generous use of protein is the cheapest nutritional insurance we can buy for children. This evidence indicates overwhelmingly that such a diet is highly beneficial.

## B OTHER STUDIES

Good accord is found in a comparison of these latest findings with those of earlier investigators in Table XII. The large standard deviations of the average nitrogen retentions for each age and body weight group leads Macy (1942) to doubt the usefulness of adjusting protein intake to either of these two parameters. This investigator feels that the relationship of nitrogen retention to nitrogen intake is a more significant index of nitrogen metabolism of the child and adequacy of the diet than any other mathematical correlation of nitrogen balance data. Macy concludes from her investigations that an average daily consumption of 473 mg of nitrogen per kilogram of body weight per day is adequate for all children of this age group. Although it is obviously difficult to determine the amino acid composition of a heterogeneous diet composed of animal and vegetable proteins, a tentative estimate of the amino acid content of the diet of a 12 year old child has been made by this worker and is compared in Table XIII with the amino acid content of an equivalent amount of cow's milk nitrogen. It appears from this tabulation that the proteins of natural food diet in these studies provided a greater intake of cystine, methionine, and lysine than do two liters of cow's milk.

As might be expected, the nitrogen retention of the growing child is seriously affected by dietary factors other than the quantity and quality of the protein component. Chaney and Blunt (1925) found that nitrogen assimilation of two growing girls, 10 and 11 years old, on diets of corn

TABLE VII  
THE PROTEIN NITROGEN INTAKE AND RETENTION OF THE PREADOLESCENTS<sup>a</sup>

Authors	No of subjects	Age in years	Average weight (kg)	N Intake (mg/kg)	N retention (mg/kg)
Porter (1939)	1	2.5	15	428	87
	1	4.7	18	374	64
	1	5.5	23	339	54
Daniels (1941)	1	3.0	19.5	451	50
	1	4.0	19.5	500	56
Daniels <i>et al</i> (1935)	8	4.4	15.4	550-590	121
	17	4.4	16.2	500-540	101
	15	4.7	16.9	450-490	83
Wang <i>et al</i> (1928a)	13	7.2	—	516	89
	10	9.9	—	418	42
Wang <i>et al</i> (1925b)	7	8.9	25.8	537	76
	7	8.9	24.9	264	1
Macy (1942)	5	4	18.4	522	37
	5	5	18.7	520	32
	6	6	21.8	466	20
	6	8	26.2	442	24
	2	9	28.4	457	37
	3	10	33.3	393	31
	1	11	35.8	371	22
	1	12	42.0	317	19

<sup>a</sup> Albanese (1947)

TABLE VIII  
COMPARISON OF THE ESTIMATED DAILY AMINO ACID INTAKE OF A 12 YEAR OLD BOY ON A MIXED DIET AND THE AMINO ACID CONTENT OF AN EQUIVALENT AMOUNT OF COW'S MILK

Amino acid	Natural food diets <sup>a</sup> (gm)	Cow's milk <sup>b</sup> (gm)
Cystine	0.94	0.58
Methionine	2.35	1.74
Arginine	4.26	2.48
Histidine	1.41	1.60
Lysine	4.16	1.11
Tyrosine	3.46	3.80
Tryptophan	0.83	1.00
Phenylalanine	3.78	3.46
Threonine	2.87	3.04
Valine	2.83	4.56
Leucine	8.65	7.12
Isoleucine	2.77	4.24
Total amino acids	38.31	34.73
Total known N intake	11.63	11.63

<sup>a</sup> Data from Macy (1942)

<sup>b</sup> Approximately 2.0 liters Calculated from Macy *et al* (1953)

parable protein content was augmented threefold by the daily administration of 600-700 ml of orange juice. In view of the observed metabolic relationship of vitamins and amino acids in man and experimental animals (Goldsmith, 1958; Wetzel *et al*, 1952; Chow, 1951; Krehl *et al*, 1945), these noteworthy experiments should be repeated and supplemented with data on urinary metabolites. Wang and associates (1928a) reported that the per cent nitrogen retention of children 6-13 years of age was greater for those underweight than those of normal body weight. These workers also observed (1928b) that the nitrogen retention of 9 undernourished children was greater than that of 8 normal children of similar age 4-12 years, on a low but not on a high protein intake. Hubbell and Koehne (1934), in a study of 17 children from 7 to 11 years of age found that the average retention of 26 mg of nitrogen per kilogram per day was not appreciably altered by the inclusion of carbohydrates to give a 6% increase in caloric value of the diet. Davis (1935) found that the nitrogen retention of children 8-11 years of age was higher on a base forming diet than on an acid forming diet even though the protein intake was slightly greater with the latter diet. Administration of cholesterol to girls of premenarchial age has been shown by Johnston (1944) to significantly increase the nitrogen retention. The nitrogen retention of children 6-8 years of age has been reported by Nothass and Schadow (1930) to be increased under the influence of sea climate with no increase in body weight. Ultraviolet irradiation has an irregular effect. Thyroid administration was found by Johnston and Maronev (1939) to increase nitrogen retention in this age group. There appears to be no marked sex differences in nitrogen retention or protein requirement for this age group (Macy, 1942; Holt and Fales 1921).

The problems involved in estimating the long term protein intakes of individual children and the possible relationship of these to the children's pattern of growth and development and to their specific needs, have long been studied by Stuart *et al* (1958). Studies on 2 boys from the age of 2-18 years have been presented in detail to illustrate these problems. One boy was able to consume levels of calories and of protein far above recommended allowances while remaining relatively inactive and maintaining low weight for height, excellent health, and a steadily high level of progress in growth. This boy became obese only for a short period late in adolescence during which his formerly high levels of intake were greatly increased. In contrast the second boy was always small and he continually consumed small intakes of both calories and protein. The protein intake was particularly low in respect to National Research Council (NRC) allowances during the ages of 6-12 years. Progress in skeletal development became slowed during this period,

health was only fair and he manifested generally lowered resistance to infections. Recognizable relationships appear in these cases between overall pattern of growth and characteristics of caloric and protein intake.

Beal (1957) reported on her findings on a longitudinal study of food patterns of 63 children from birth to 7 years of age whose patterns of growth are thought to be satisfactory. The intake level of specific nutrients are based on nearly 1,400 nutrition histories. Typical variations

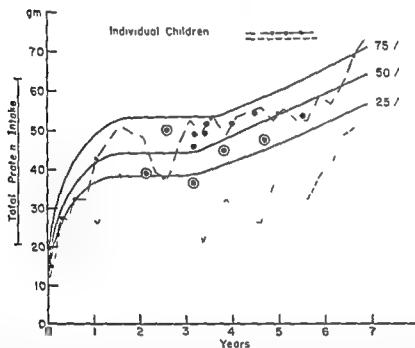


FIG. 12. Intake of protein in the first 7 years of life showing patterns of 2 individual children contrasted with the 25th, 50th and 75th percentiles of the group from Beal (1957). The dots show the data obtained with children in our hospital. The circled dots show the data of children given lysine supplements.

of protein intake with age found by this investigator are shown in Fig. 12. Attention was called to the fact that only one child in this series has not had a decrease in milk intake during the preschool period. The factors operating in this decrease in milk intake are at the present time subject to speculation. The rate of growth is less rapid during late infancy and the preschool period than during early infancy. Lynch and Snively (1951) have pointed out that it is almost impossible for the toddler and preschool child to have a satisfactory feeding program when milk intake exceeds one pint daily.

Dietary measurements on 12 children 2.2-5.5 years of age were done in our Institution for periods of 12-67 days. The observed average

protein intake of these children has been compared graphically with Beals data (Fig 12) Very good agreement occurs between our findings and those of Beal, at the 50th percentile, throughout this age range It is significant that the NRC allowances are lower than Beal's 25th per

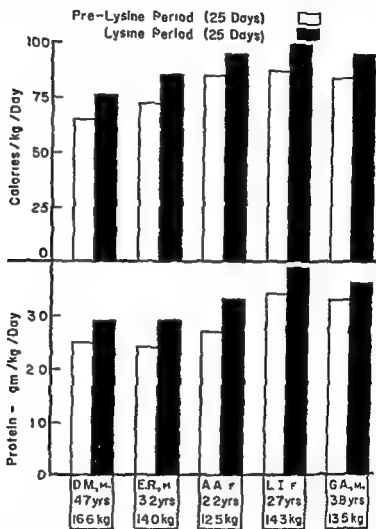


FIG 13 The effect of lysine fortification on caloric and protein intake of young children

centile group The effect of lysine supplementation (1 gm per day for 25 days) on the caloric and protein intake of 5 of our young subjects who fell within the 25th 50th and 75th percentile groups was studied The findings are recorded in Fig 13 The period of lysine supplementation was associated with an increased average daily intake of calories and proteins in all 5 children



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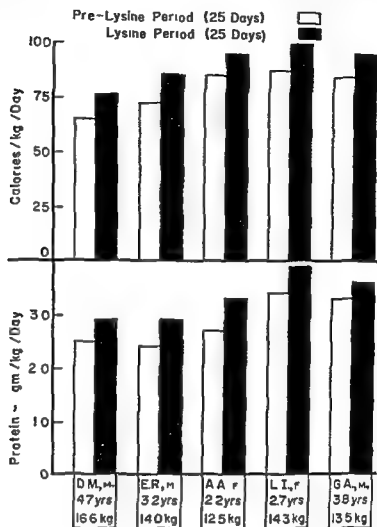


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### III ADOLESCENCE

#### A HEALTHY CHILDREN

The subject of the protein intakes of adolescent boys and girls was reviewed by Holt and Fales in 1921. The data from their own investigations of healthy children 10-17 years of age, from intelligent, well to do families are shown in Fig 14. It is to be noted that the protein intakes, consisting of two thirds animal protein and one third vegetable protein are generally higher for the boys and especially so in the tenth, thirteenth, and fourteenth years. Since the protein intake of these studies was regulated by appetite alone the values do not represent minimum figures. However the record of these protein values for adolescent girls

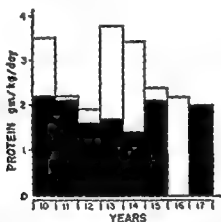


FIG 14 Comparison of protein intake of adolescent boys and girls. This chart was constructed from the data reported by Holt and Fales (1921). No suitable values are available for boys in the seventeenth year and for girls in the sixteenth year. Solid areas represent data for girls (Albanese 1917).

with those of Wait and Roberts (1933) which are based on nitrogen balance data indicate that appetite constitutes a good criterion of this dietary need. The report of Wait and Roberts which reviews the earlier literature and presents their own data on 52 healthy girls from 10 to 16 years of age shows that of the 13 balances on normal children of 10 to 11 years all intakes below 2 gm of protein per kilogram resulted in a loss of nitrogen from the body while storage was achieved on protein intakes of 2.57-3.08 gm per kilogram. These investigators found no close relation between the required total protein and any of the single variants age, weight and height. When combinations of three rather than merely two of these bases are used the correlations are higher with the exception of height referred to age and the calculated figure closely approximates the observed results.

Investigations of Hsu and Adolph (1940) with two groups of boys



14-16 years of age one fed on a white bread diet supplemented with dried cows or soybean milk, and the other group maintained on a mixed cereal diet, disclosed that the nitrogen retention could be raised from 50 mg of nitrogen per kilogram to 80 mg of nitrogen per kilogram by increasing the protein intake from 1.68 to 1.90 gm per kilogram. This indicates that lower protein intake was suboptimal.

### B. PROTEIN NEEDS IN DISEASE

Johnston (1958) has recently reviewed his extensive studies on the problem of optimal intake of protein for adolescents. In following for more than twenty years a group of 932 tuberculin reactors removed from contact, it was possible to demonstrate good correlation between the development of these lesions, their course when once developed, and the nitrogen metabolism. Negative nitrogen balances, or uncompensated previous protein depletion from any cause were associated with spread of the disease process. In particular, Johnston repeatedly found that sexual maturation in girls, which is not necessarily coincidental with the onset of menstruation, carries with it a depression in nitrogen retention. Failure to increase the dietary protein intake indicated by this fall in nitrogen storage favors the spread of tuberculosis in girls with minimal reinfection type of lesion.

Findings on the adolescent needs for calories and protein are collected in Table XIV. It is to be noted that the percentage of calories from protein found in recent dietary surveys as well as those recommended by the NRC, are lower than the percentage reported by Johnston and Maroney (1939), Wang and associates (1936), Holt and Fales (1921) and Wait and Roberts (1933). Johnston's metabolic balance data (1958) suggest that optimal intake of protein for the adolescent should constitute at least 15% of an adequate caloric intake (basal calories  $\times 1.65$ ) and that the NRC recommendations for this age group be revised upward. Johnston urges that one criterion of optimal should be the relation of a nutrient to infection. Support for this view is afforded by the findings of Coburn and Moore (1943) that the ultimate prognosis of rheumatic fever in children can be correlated with nutritional state with particular reference to protein reserves.

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## CHAPTER 15

# Amino Acid Requirements of Young Adults

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## I INTRODUCTION

Purified amino acids and nitrogen balance methodology have made it possible to study the qualitative and quantitative amino acid requirements of man. These tools in the hands of specialized research workers together with the cooperation of many young men and women who served as subjects have yielded much basic information on amino acid requirement during the last two decades and promise to yield still more in the future.

Threonine was the last of the essential amino acids to be isolated and identified. When its nature and importance were known mixtures of amino acids including threonine could be used in place of proteins to support the growth of rats. Quantitative studies followed the qualitative ones and then studies with human subjects followed those with animals.

## II BACKGROUND

### A CLASSIFICATION OF AMINO ACIDS

In 1941 Holt and his co-workers reported the necessity of supplying certain amino acids preformed in the food in order to maintain human subjects in nitrogen equilibrium. Negative nitrogen balances inevitably occurred when the diet was deficient in tryptophan (Holt *et al* 1941) or lysine (Albanese *et al* 1941) or methionine (Albanese *et al* 1943). Nitrogen equilibrium was re-established when the missing acid was added.



In 1942, Rose *et al* published a note stating that valine and isoleucine were indispensable for the maintenance of nitrogen equilibrium. In 1943, he added threonine, leucine, and phenylalanine to this list of essential amino acids for man.

In 1944, Albanese *et al* reported that methionine alone could supply the need for sulfur containing amino acids, and cystine was retired to the group of dispensable amino acids for man. Arginine and histidine were found to be dispensable also (Holt *et al*, 1942, Rose *et al*, 1943).

The complete list of essential amino acids—those which must be supplied preformed to maintain nitrogen equilibrium in man—was given by Dr. Rose in 1949 and included isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan, and valine.

The classification of essential or indispensable for these amino acids and "nonessential or dispensable" for all of the other amino acids does not refer to the body's need for the different acids but only to the body's need to have certain ones supplied preformed. Amino acids which can be formed within the body, when the appropriate materials are supplied, have important functions and are essential. Their classification as nonessential can be misleading, especially in nontechnical circles.

## B. QUANTITATIVE STUDIES—MEN

The studies of the quantitative requirement for these amino acids which followed their identification called for a relatively new idea in human feeding studies—the use of purified or semi-synthetic diets. In such diets pure chemicals supplied the minerals and vitamins; amino acids were used in place of proteins; and sucrose, cornstarch, and butter fat were the chief sources of energy. Only in this way could the composition of the diet be kept constant while a single amino acid was quantified.

Tryptophan was the first amino acid for which quantitative results were reported from studies of human subjects (Holt *et al*, 1944, and Baldwin and Berg 1949). However, from 1942 through 1952, Rose and his associates at the University of Illinois were making a systematic study of the requirement of young men for each of the 8 essential amino acids. In 1949 he (Rose, 1949) reported tentative values for minimum requirements and recommended intakes of these acids. In the detailed reports the term "recommended intake" was changed to safe "intake" (Rose *et al*, 1954) but by either designation it was twice the minimum requirement. The minimum requirement was the amount needed by the subject who required the largest quantity of an amino acid to maintain a "slight but distinctly positive nitrogen balance as measured by the average for a period of several days." A thorough recounting of these

important and now published results has been made recently by Rose (1957) and is recommended to the reader

### III QUANTITATIVE STUDIES OF YOUNG WOMEN

Early in the 1950s plans were made jointly by research workers in human nutrition in the United States Department of Agriculture and in the Home Economics departments of three universities to study the amino acid requirements of young women. The research leaders their university locations and the amino acids they studied were: Dr Ruth Leverton at the University of Nebraska studied threonine valine tryptophan, phenylalanine with and without tyrosine and leucine; Dr Marian Swendsen at the University of California (Los Angeles) studied methionine and cystine and isoleucine; and Dr May Reynolds at the University of Wisconsin studied lysine and methionine and cystine. The research studies were supported in part by U. S. Department of Agriculture contracts sponsored by the Human Nutrition Research Division of the Agricultural Research Service. In addition Dr Helen Clark at Purdue University has investigated the lysine requirement of both men and women as one phase of the North Central regional project on the nutritional status of adults.

#### A PROCEDURE

##### 1 General Principle

The procedure used by Leverton and her associates at the University of Nebraska will be discussed in some detail because it was also followed in general by the other researchers. The method was patterned after that of Rose (1949) and was based on the fact that when one or more essential amino acid is absent from the diet or present in insufficient amounts the nitrogen excretion exceeds the intake and a negative nitrogen balance occurs. To determine the quantitative requirement for an essential amino acid it was necessary to find the least amount of that acid which would maintain nitrogen equilibrium in the subjects when the other essential amino acids were present in adequate amounts.

##### 2 Plan of Studies

The first step in the experimental procedure was to feed each subject a ration adequate in the known dietary essential and generous in all of the essential amino acids until she had been in nitrogen equilibrium for at least 7 days. The second step was to reduce stepwise the intake of the amino acid to be measured until the subject went into negative nitrogen balance for at least 4 days. Finally the intake was then increased stepwise until nitrogen equilibrium was again established. The least amount

which maintained a subject in nitrogen equilibrium was regarded tentatively as her requirement.

The other investigators chose to reduce the quantity of the amino acid sharply or omit it entirely after the subjects had established nitrogen equilibrium on the complete mixture of amino acids. This induces negative balance more quickly than reducing the intake gradually.

Five separate studies were conducted at the University of Nebraska between September 1951 and April 1954. The major part of each study period was devoted to work with one amino acid. Following this exception in the first study there was an ancillary study to repeat some level of a amino acid previously studied or to try a level of one which was to be quantified later.

After the requirement for each of the 5 amino acids threonine, valine, tryptophan, phenylalanine, and leucine had been studied separately they were all fed together with the other acids in the amino acid ration, in the least amounts which had been found capable of maintaining nitrogen equilibrium in all of the subjects. This was called the Test Mix ration. In this way the adequacy of the minimum amount of each of the 5 amino acids measured separately was tested in the presence of minimum amounts of the other four.

Finally in the last study made by Swendseid and Dunn (1956) it was possible for her to feed minimum amounts of all 8 of the essential amino acids.

### 3 Subjects

In the Nebraska studies 35 college girls who ranged from 19 to 26 years of age served as subjects (one of them served in two studies). Fifteen similar subjects in the California studies ranged in age from 20 to 36 years. In the Wisconsin studies the 24 women subjects (2 of them served in each of two studies) were both students and staff members. 12 subjects were from 18 to 29 years, 9 subjects were from 30 to 31 years, and 3 subjects were 43, 47, and 64 years of age.

At the beginning and end of the studies each subject had a medical examination including routine laboratory tests of blood and urine. All of the subjects were considered healthy, within normal limits of weight for height, and had metabolic rates within the normal range for their ages.

### 4 Preliminary Period

During the first days of each study the subjects were given a weighed diet composed of ordinary foods. The kind and amount of food were the same each day except for the amounts of fat and sugar which were

adjusted to meet the caloric requirements of the individual subjects. This period gave the subjects time to adjust physiologically and psychologically to the experimental set up which was to become more rigid and increasingly tiresome. The subjects usually came into nitrogen equilibrium during this period of controlled food intake which varied in length from 8 to 14 days in the different studies.

### 5 Experimental Period

The experimental period included all the days following the preliminary period until the end of the study. The diet was semi purified and included a limited number of foods which were extremely low in nitrogen, mineral and vitamin supplements and amino acids in purified form.

**Amino Acids.** The amounts of the 12 amino acids, which were fed daily in the same ratio as they occur in whole egg protein (Mitchell and Block 1946) and the nitrogen supplied by them are shown in Table I. Egg protein was chosen as the reference or pattern for the amounts of the different amino acids to be fed because of its recognized high biological value. The amino acids were tested for purity by nitrogen analysis, optical rotation and microbiological assay. The L form of each of the acids except isoleucine was used. DL isoleucine was used.

All of the amino acids except cystine, tyrosine and the one being tested were ground together in a ball mill. The mixture was then analyzed for nitrogen to check its total nitrogen content against the sum of the results of the nitrogen analysis of each component.

The total daily nitrogen intake was 6.2 gm for the first threonine study, 8.5 and 9.5 gm for the valine study and for repeating certain levels of threonine and 9.5 gm for the last three studies. Glycine or glycine plus diammonium citrate was the chief source of nitrogen and made up the difference between the nitrogen supplied by the 12 amino acids and the desired nitrogen intake.

The level of total nitrogen used in the California studies was 6-7 gm and in the Wisconsin studies 10-11 gm. In the study of the sulfur containing amino acids Swendsen supplied a portion of the amino acids from 2 gm of nitrogen as peanut protein and added purified amino acids to bring the intake up to the equivalent of 20 gm of egg protein.

Each subject's daily portion of the amino acid mixture was divided into three portions and one portion was fed at each of the three meals. The appropriate amount was weighed into a tumbler and dissolved in water prior to mealtime. The amino acid being tested was in a separate solution and was added to this solution. The diammonium citrate solution was also added to the dissolved acids. Because of their relative in

5 ml containing 10 USP antipernicious anemia units was given every third day during the first four studies. During the leucine study, however, 0.5 gm of liver concentrate 120 was given daily. Mixed tocopherol acetates, 38 IU, were given every third day during the last two studies. The vitamin concentrates which were used in the three locations differed slightly but in each study supplied ample amounts of those known to be required.

TABLE II  
COMPOSITION OF MINERAL SUPPLEMENT<sup>a</sup>

Salts	Amount of element supplied (gm./subject/day)	
Mineral mix		
CaCO <sub>3</sub>	0.548	Ca
KH <sub>2</sub> PO <sub>4</sub>	0.298	P
MgCO <sub>3</sub> · 6H <sub>2</sub> O	0.199	Mg
FeC <sub>6</sub> H <sub>5</sub> O <sub>7</sub> · 6H <sub>2</sub> O	0.015	Fe
CuSO <sub>4</sub> · 5H <sub>2</sub> O	0.002	Cu
KI	0.00015	I
MnCl <sub>2</sub> · 4H <sub>2</sub> O	0.002	Mn
ZnCl <sub>2</sub>	0.0009	Zn
Baking powder <sup>b</sup>		
NaHCO <sub>3</sub> +		
Ca(H <sub>2</sub> PO <sub>4</sub> ) <sub>2</sub> · H <sub>2</sub> O	0.455	Ca
	0.703	P

<sup>a</sup> Leverton *et al.* (1956a)

<sup>b</sup> Rose *et al.* (1950) also included 2.69 gm. cornstarch

*d. Caloric Intake.* Calculated daily caloric intakes during the experimental periods ranged from 2,200 to 2,900 or from 35 to 54 calories per kilogram of actual body weight. The mean daily caloric intake for the 36 subjects was 2,500 calories or 42 calories per kilogram.

It was recognized that caloric requirements would probably be increased when the subjects were on the semi-purified diet. Therefore much attention was given to preventing the subjects from losing weight and also in preventing them from gaining more than 2 or 3 pounds in weight during the studies. The subjects were weighed daily before breakfast and caloric intakes were adjusted upward or downward as seemed necessary. However the caloric intake was kept as high as possible without causing a gain of more than 2 or 3 pounds during any study. It was just under the amount that would have resulted in a definite gain. On the basis of previous experience we knew that this level of caloric intake was considerably higher than one which was just sufficient to prevent weight loss. Keeping the caloric intake this high

helped to prevent the oxidation of body tissue for energy and a replacement of this tissue with water without a noticeable change in body weight. However maintenance of weight is not as sensitive a measure of caloric requirement as would be desirable for such rigidly controlled studies of nitrogen balance. A very practical aspect of this concern with energy intake in any metabolism study is the attitude of subjects themselves. They will not volunteer for a study which will result in a weight gain over a period of 50 to 60 days. If they begin to gain weight during a study they become apprehensive and uncooperative, and may even withdraw from the study.

The mean caloric intake of the Nebraska subjects was higher than for the group of Wisconsin subjects who needed a mean intake of only 2286 calories or 37.8 calories per kilogram to maintain constant weight.

In methionine cystine studies at California 7 of the 8 subjects maintained weight during the period of the semi synthetic diet on the same caloric intake as they had had during the preliminary period when ordinary foods supplied a mean daily intake of 2030 calories or 34.6 calories per kilogram. One subject had required additional calories. During the study of isoleucine however the subjects caloric intake had to be increased above what they had had in the preliminary period. During the semi synthetic diet regimen their caloric intake averaged approximately 2520 calories per day or 43.6 calories per kilogram.

### 6 *Metabolism Study and Nitrogen Balance*

Urine and feces were collected for the entire period of study from the first day of the preliminary period through the last day of the semi purified diet. Urine was acidified except during the tryptophan study and refrigerated under toluene until a 24 hour collection was complete. Nitrogen and creatinine determinations were done on the urine within 4 hours after the completion of each 24 hour collection.

Carmine was given to mark the feces when the level of amino acid intake was changed. The fecal collections were frozen until combined into a composite for a period and then made into a digest with 20% HCl for nitrogen analysis.

Nitrogen was determined by the macro Kjeldahl method (Assoc. Offic. Agr. Chemists 1950 p. 13) and the ammonia was collected in boric acid (Markley and Hann 1925). Creatinine was determined by the Folin method using picric acid (Assoc. Offic. Agr. Chemists 1950 p. 368) and the intensity of the color was measured photometrically.

Different subjects were not always on each level of amino acid intake the same number of days but in general for 6 days or longer. The subjects who had the greatest daily variation in urinary nitrogen were kept

on each level of intake longer than the subject who showed little variation. In a few cases the time had to be reduced to less than 6 days because symptoms of ill health in the subjects were suspected, or too severe negative nitrogen balances occurred.

The nitrogen balance, or the intake minus the excretion was calculated for each subject each day as the study progressed. The cumulative nitrogen balance of each subject as she continued on a given intake, was reviewed daily and used as the basis for deciding when to change the level of amino acid intake and whether to increase or decrease it. Because of the time lag in securing the values for the fecal nitrogen, the figures from the preceding period were used temporarily to add to the analyzed urinary values.

Because of the inherent variations, both human and technical in human metabolism studies the Nebraska authors have preferred to define nitrogen equilibrium as a zone in which the excretion and intake closely approximate each other, rather than a single point at which they are numerically identical. In these studies of amino acid requirements, therefore nitrogen equilibrium is defined as the zone in which the difference between the intake and excretion does not exceed  $\pm 5\%$ , i.e., the excretion is within 95-105% of the intake. Nitrogen loss from the body ordinarily referred to as negative balance is considered to exist when the excretion is more than 105% of the intake conversely nitrogen retention or positive balance is considered to exist when the excretion is less than 95% of the intake. The reader, however need not be limited by this interpretation. The results are presented in sufficient detail to permit him to use any criterion he prefers for judging nitrogen equilibrium and minimum requirements.

Studying the amino acid requirements from the standpoint of the minimum amounts needed for maintaining nitrogen equilibrium was not meant to imply that nitrogen storage was not important for young women in the age range of many of these subjects. It seemed however that nitrogen equilibrium rather than nitrogen storage would come closer to being a common denominator to the nitrogen metabolism of all of the subjects.

## B RESULTS

1 *Threonine Valine Tryptophan Phenylalanine, and Leucine*  
The data for the five studies have been reported by Leverton *et al* (1956a-e). The mean daily nitrogen balances of each subject on the various levels of intake of each of the amino acids studied are charted in Figs 1 to 5. The performance of each subject may be followed as her intake was changed from one level to another. The solid line joins the





nitrogen balance the standard deviation and the range in the balances for each group of subjects on each level of these different amino acids. Also shown are the number of subjects with nitrogen excretions which are (1) greater than 105% of the intake, and (2) greater than 100% of the intake.

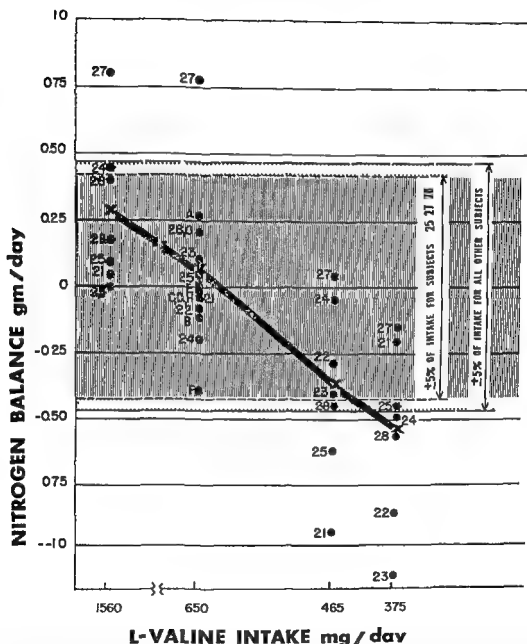


FIG. 2. Valine intakes and nitrogen balances of 15 subjects for 255 subject days. Each subject is identified by her code which appears beside her retention for each level on which she was studied. X = mean retention of all subjects on each level of intake. Intake includes 100 mg/day supplied by auxiliary foods. (From Leverton *et al.* *J. Nutrition* 58: 86, 1958.)

The results of an auxiliary study of different levels of tyrosine and phenylalanine are shown in Fig 6 and Table IV

In the Nebraska studies two features of the nitrogen balances of the subjects were considered in suggesting tentative minimum requirements for each of the 5 amino acids studied (1) whether the nitrogen balances of the subjects were in the zone of equilibrium (or positive) and (2) whether the subjects had significantly better nitrogen balances when the next higher level of intake was fed Table V has shown the nitrogen

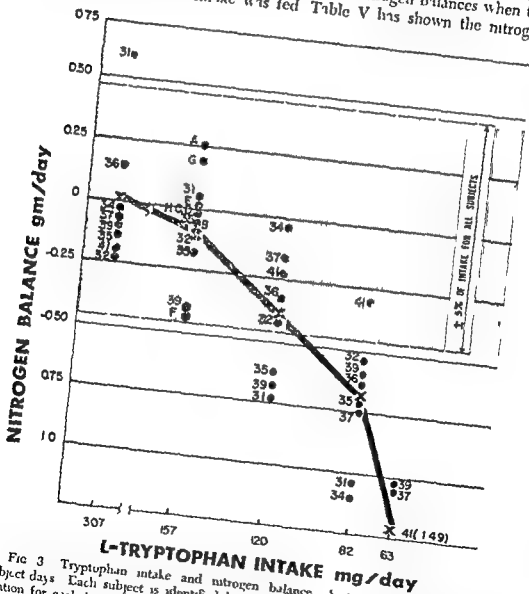


FIG 3 Tryptophan intake and nitrogen balance. Sixteen subjects for 285 subject days. Each subject is identified by her code which appears beside her retention for each level on which she was studied.  $\bar{x}$  = mean retention of all subjects on each level of intake. Intake includes 7 mg/day supplied by auxiliary foods. (From Leverton et al. *J Nutrition* 58: 222, 1958.)

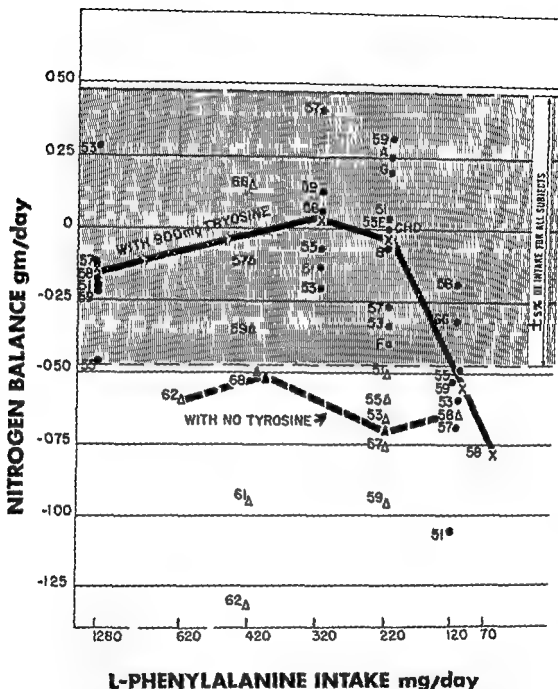


FIG 4 Phenylalanine intake and nitrogen balance. Each subject is identified by her code which appears beside her retention for each level on which she was studied. Intake includes 20 mg/day of phenylalanine from auxiliary foods. ● Represents subjects receiving 900 mg of tyrosine mean = X Δ Represents subjects receiving no tyrosine mean = Δ (From Leverton *et al* J Nutrition 58 344 1958)

balances of the group and the number of subjects in nitrogen equilibrium Table V shows (1) the level of intake of each acid which yielded a significantly better mean daily nitrogen balance than the levels below it, and (2) the level above which there was no significantly better mean daily nitrogen balance

When the subjects were receiving 214 mg threonine daily the mean nitrogen balance was significantly better than when the intake was 103

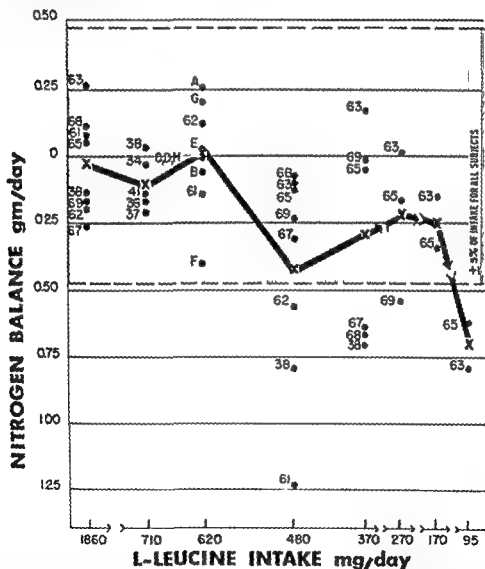


FIG 5 Leucine intake and nitrogen balance 13 subjects for 334 subject-days. Each subject is identified by her code which appears beside her retention for each level on which she was studied.  $\lambda$  = mean retention of all subjects on each level of intake. Intake includes 20 mg/day supplied by auxiliary foods (From Leverton et al., *J Nutrition* 58: 358, 1956)

mg but when the intake was 305 mg daily, the balance was not significantly better than on an intake of 214 mg. However, when 8 subjects were placed on the Test Mix which supplied 214 mg threonine, 2 subjects were in negative balance until their threonine intake was increased to 305 mg. For this reason 305 mg, rounded to 310 mg, rather than 214

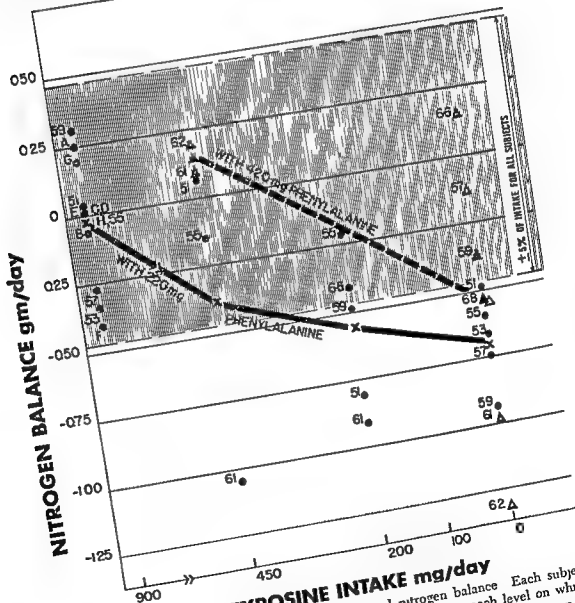


FIG 6 Tyrosine and phenylalanine intake and nitrogen balance. Each subject is identified by her code which appears beside her retention for each level on which she was studied. ● Represents subjects on 220 mg of phenylalanine mean =  $\bar{x}$ . ▲ Represents subjects on 420 mg of phenylalanine mean =  $\bar{x}$ . (From Levertton et al. J Nutrition 58:349, 1956)

TABLE III  
MEAN NITROGEN BALANCES OF SUBJECTS ON DIFFERENT LEVELS OF 5 AMINO ACID INTAKES<sup>a</sup>

MEAN NITROGEN BALANCES OF SUBJECTS ON DIFFERENT LEVELS OF 2-AMINO ACID INTAKE									
Level of intake (mg/day)	Number of subjects on each level	Total number of subject days	Nitrogen balance			Number of subjects with nitrogen excretions			
			Mean (gm/day)	SD <sup>b</sup>	Range Low (gm/day) High (gm/day)	Greater than 105% of intake <sup>c</sup>	Equal to or less than 100% of intake <sup>c</sup>		
Threonine									
62 gm N/day									
765	6	55	0.10	0.21	-0.10 0.51	0	4		
397	6	32	0.06	0.22	-0.28 0.33	0	3		
214	5	25	-0.11	0.21	-0.30 0.16	0	2		
103	6	42	-0.49	0.26	-0.76 -0.12	4	0		
0	6	66	-0.81	0.19	-1.05 -0.53	6	0		
147 DL (73.5L)	5	32	-0.73	0.21	-1.07 -0.52	5	0		
85 or 95 gm N/day									
580	7	96	0.14	0.32	-0.20 0.78	0	4		
305	4	18	-0.04	0.11	-0.17 0.03	0	2		
214	5	34	-0.21	0.41	-0.71 0.35	2	1		
Valine									
1560	7	55	0.27	0.29	0.00 0.80	0	7		
650	7	69	0.11	0.31	-0.20 0.76	0	4		
465	7	48	-0.39	0.34	-0.95 0.03	3	1		
375	7	31	-0.55	0.35	-1.16 -0.17	2	0		

<sup>a</sup> Leverton *et al.* (1956a-e)<sup>b</sup> Standard deviation<sup>c</sup> Negative balance<sup>d</sup> Equilibrium or positive balance

TABLE III (Continued)

TABLE III (Continued)									
Level of intake (mg/day)	Number of subjects on each level	Total number of subject days	Nitrogen balance			Number of subjects with nitrogen excretions			
			Mean (gm/day)	S D <sup>b</sup> (gm/day)	Range Low (gm/day) High (gm/day)	Greater than 105% of intake <sup>c</sup>	Equal to or less than 100% of intake <sup>d</sup>		
<b>Tryptophan</b>									
307	8	56	0.01	0.26	-0.21 0.59	0	2		
157	5	38	-0.13	0.16	-0.39 0.05	0	1		
120	8	73	-0.39	0.26	-0.73 -0.04	3	0		
82	8	56	-0.70	0.26	-1.10 -0.30	7	0		
63	3	11	-1.19	0.26	-1.49 -1.02	3	0		
<b>Phenylalanine (With 900 mg tyrosine)</b>									
1250	6	45	-0.15	0.24	-0.45 0.27	0	1		
320	6	38	0.03	0.22	-0.21 0.41	0	3		
220	5	43	-0.05	0.26	-0.33 0.32	5	0		
120	7	56	-0.56	0.26	-1.07 -0.18	1	0		
70	1	5	-0.76	0.46	-1.17 0.02	1	0		
<b>(With no tyrosine)</b>									
620	1	5	-0.59	0.34	-1.00 -0.17	1	1		
420	6	38	-0.52	0.54	-1.31 0.16	3	0		
220	5	34	-0.69	0.17	-0.94 -0.50	5	0		
120	1	7	-0.63	0.46	-1.53 -0.13	1	0		

TABLE III (Continued)

Level of intake (mg/day)	Number of subjects on each level	Total number of subject days	Nitrogen balance			Number of subjects with nitrogen excretions		
			Mean (gm/day)	S D <sup>a</sup>	Range Low (gm/day) High (gm/day)	Greater than 105% of intake <sup>c</sup>	Equal to or less than 100% of intake <sup>d</sup>	
Leucine								
1860	8	57	-0.03	0.18	-0.26	0.25	0	4
710	5	39	-0.12	0.20	-0.27	0.46	0	1
620	2	12	-0.01	0.52	-0.14	0.12	0	1
480	8	88	-0.43	0.41	-1.35	-0.08	3	0
370	6	43	-0.32	0.39	-0.70	0.17	3	1
270	3	21	-0.23	0.29	-0.54	0.01	1	1
170	2	11	-0.25	0.13	-0.34	-0.15	0	0
95	2	11	-0.71	0.12	-0.79	-0.62	0	0
Test mix								
(Minimum of all 5 acids)	8	52	0.00	0.19	-0.40	0.25	0	8



TABLE IV  
MEAN NITROGEN BALANCES OF SUBJECTS ON DIFFERENT LEVELS OF TYROSINE AND PHENYLALANINE INTAKES<sup>a</sup>  
Number of subjects with nitrogen excretions

MEAN NITROGEN BALANCES OF SUBJECTS ON DIFFERENT LEVELS OF EXCRETIONS									
Level of L-phenylalanine (mg./day)	Level of L-tyrosine (mg./day)	Number of subjects on each level	Total number of subject days	Nitrogen balance			Greater than 105% of intakes <sup>d</sup>	Equal to or less than 100% of intakes <sup>d</sup>	
				Mean (gm./day)	SD <sup>a</sup>	Range			
						Low (gm./day)			High
220	200	5	28	-0.56	0.29	-0.89	-0.21	3	0
420	450	2	10	0.12	—	0.06	0.18	0	2
220	450	3	15	-0.38	0.59	-1.05	0.08	1	1
* (1056d)									

<sup>a</sup> Leverton et al (1958d)

<sup>b</sup> Standard deviation

<sup>c</sup> Negative balance

<sup>d</sup> Equilibrium or positive balance

mg threonine is the amount which has been suggested as a tentative minimum requirement for young women similar to those studied here

In the case of valine, tryptophan, phenylalanine with tyrosine, and leucine the level of intake which resulted in a significantly better nitrogen balance than the levels below it was also the level above which there was no significantly better balance. These levels were 650 mg valine, 157 mg tryptophan (rounded to 160 mg), 220 mg phenylalanine with 900 mg tyrosine and 620 mg leucine. They have been suggested as tentative minimum requirements for young women.

TABLE V

STATISTICAL SIGNIFICANCE OF DIFFERENCE IN NITROGEN BALANCE ON DIFFERENT INTAKES

Amino acid	Levels of amino acid intake (mg/day) between which differences in nitrogen balances were		
	Significant		Not significant
Threonine	214 and 103	5%*	214 and 305 or higher
Valine	650 and 465	1%	650 and 1580
Tryptophan	157 and 120	1%	157 and 307
Phenylalanine with 900 mg tyrosine	220 and 120	1%	220 and 320 or higher
Leucine	620 and 480	1%	620 and 710 or higher

\* Level of probability

## 2 Isoleucine

The results of the study of isoleucine (Swendseid and Dunn 1956) are shown in Table VI in terms of the mean daily nitrogen balances of each group of subjects in each level of intake, the range in nitrogen balances and the number of subjects in equilibrium. The amounts of isoleucine required for nitrogen equilibrium varied from 250 to 450 mg. 3 of the subjects required 250 mg, 3 required 350 mg, and 1 required 450 mg. When these figures are evaluated in the same way as the Nebraska results, the highest figure of 450 mg isoleucine may be suggested as the minimum requirement for young women.

## 3 Lysine

In the Wisconsin studies (Jones *et al.* 1956) 14 women were studied on different levels of lysine intake and the results for the last 4 days of each intake period are summarized in Table VII. Daily intakes of 250 mg and below resulted in consistently negative balances among the subjects, although in a few cases the nitrogen excretion was not more than 105% of the intake. On a lysine intake of 400 mg only 1 subject was in serious negative balance and she required 500 mg for equilibrium. Nitrogen equilibrium or storage occurred on intakes above 500 mg ex

cept for subject ■ Her nitrogen balance was no better on an intake of 640 mg than on 250 mg,  $-0.40$  gm, and  $-0.38$  gm, respectively, both less than 5% of the intake Her balance rose to  $-0.12$  on an intake of 1600 mg

TABLE VI  
MEAN NITROGEN BALANCES OF SUBJECTS ON DIFFERENT LEVELS OF METHIONINE AND ISOLEUCINE INTAKES<sup>a</sup>

Level of intake (mg/day)	Number of subjects on each level	Nitrogen balance			Number of subjects with nitrogen excretions	
		Mean (gm/day)	Range		Greater than 105% of intake <sup>b</sup>	Equal to or less than 100% of intake <sup>c</sup>
			Low (gm/day)	High (gm/day)		
Methionine <sup>d</sup>						
800	8	0.05	-0.18	0.43	0	3
150	8	-0.14	-0.70	0.31	3	4
250	3	0.14	-0.57	0.61	1	2
350	1	0.09	—	—	0	1
Isoleucine <sup>e</sup>						
1330	7	0.07	0.16	0.63	0	3
50	7	-1.08	-1.49	-0.81	7	0
150	3	-0.32	-0.66	-0.06	1	0
250	7	-0.49	-1.18	0.06	4	1
350	4	-0.07	-0.66	0.38	1	2
450	1	-0.18	—	—	0	0

<sup>a</sup> Swendsen *et al.* (1956)

<sup>b</sup> Negative balance

<sup>c</sup> Equilibrium or positive balance

<sup>d</sup> All diets included 200 mg L-cystine daily

<sup>e</sup> Number of days on each intake varied from 6 to 12

<sup>f</sup> Number of days on each intake varied from 6 to 12 for the 1330-mg level to 3 or 4 for the 50 mg level

<sup>g</sup> This is the subject who was next given a 450 mg intake

Clark and her co-workers (1957) studied the lysine requirements of 5 women and 5 men on a diet in which approximately one half of the daily nitrogen intake of 90 gm was supplied by wheat flour cornmeal, and a few fruits Purified amino acids were added to make the total intake of essential amino acids (plus arginine histidine, cystine, and tyrosine) equal to the amounts present in 20 gm whole egg protein, and diammonium citrate was used as the source of additional nitrogen

The amount of lysine fed was varied from 1500 mg, the amount in

20 gm whole egg protein, down to 470 to 510 mg which was present in the unsupplemented basal diet. The subjects (who ranged from 23 to 29 years of age) were treated individually in regard to the quantity of lysine tested in different 6 day periods and the results are reported only for the minimum levels of intake on which the subjects maintained nitrogen equilibrium. Equilibrium has been defined by Clark as

TABLE VII  
MEAN NITROGEN BALANCES OF SUBJECTS ON DIFFERENT LEVELS OF LYSINE INTAKE<sup>a</sup>

Level of intake (mg/day)	Number of subjects on each level	Nitrogen balance <sup>b</sup>			Number of subjects with nitrogen excretions	
		Mean (gm/day)	Range		Greater than 105% of intake <sup>c</sup>	Equal to or less than 100% of intake <sup>d</sup>
			Low (gm/day)	High (gm/day)		
Lysine						
1600	14	0.45	-0.12	1.27	0	13
100	6	-0.62	-0.88	-0.23	6	0
180	5	-0.84	-1.42	-0.41	4	0
220	5	-0.36	-0.53	-0.80	1	0
250	7	-0.46	-1.16	-0.04	3	0
400	9	0.00	-0.79	0.59	1	4
500	2	0.04	0.00	0.07	0	2
640	5	0.00	-0.40	0.29	0	4

<sup>a</sup> Jones *et al.* (1956)

<sup>b</sup> These figures are the means for the final 4 days on each lysine intake

<sup>c</sup> Negative balance

<sup>d</sup> Equilibrium or positive balance

when nitrogen balance was at or near zero. The intakes and nitrogen balances of the 5 women subjects are given in Table VIII. The amount of lysine required by these women varied from 500 to 700 mg daily. Three of the men subjects required more than 700 mg lysine daily. The relationship of lysine need to body weight surface area which was noted in the report has not been found in subsequent studies (Clark 1958).

On the basis of the results of the Wisconsin studies of lysine 500 mg daily may be considered tentatively as adequate for nitrogen equilibrium and referred to as a suggested minimum requirement for normal women. This amount would be raised to 700 mg on the basis of the results of Clark. However use of the results of the latter for comparison and

appetite when their intake of valine was reduced to 373 mg daily, 3 subjects lost an evening meal

**Tryptophan** The 3 subjects who received only 63 mg of tryptophan daily for 3 or 4 days experienced considerable nausea. One lost a meal, one had diarrhea, and one complained of extreme fatigue. During the 7 days she was on 120 mg of tryptophan, another subject lost two meals (she was not nauseated but attributed the loss of meals to a muscle spasm)

**Phenylalanine** None of the subjects showed or reported any symptoms which might have suggested an inadequate intake of a dietary essential

**Leucine** When 4 subjects were in negative nitrogen balance they had a few symptoms of gastrointestinal distress which may have been attributable to an inadequate intake of a dietary essential. In one case a meal was lost

**Isoleucine** When isoleucine was removed from the amino acid supplement of the diet all of the subjects experienced extreme discomfort. Their symptoms included loss of appetite, a feeling of nausea, dryness of skin and mucous membranes, easy fatigue, and headaches. The addition of isoleucine restored their sense of well being.

**Lysine and methionine** No evidence was noted of any discomfort or distress experienced by the subjects on low intakes of either lysine or methionine.

## 7 Need in Relation to Size

Within the size and weight range of the groups of women selected for these studies there was no discernible relationship between the amount of an amino acid required by a subject for nitrogen equilibrium and her total weight or her metabolic size, ( $\text{kg}^{.75}$ ). Probably such a relationship cannot be measured with the macrobiological methods we now employ. It is difficult to believe that the requirement for a nutrient which is essential to every cell bears no relation to the quantity or mass of those cells. However, we can hardly expect to measure the complexities of the dynamic state of protein and amino acids in the body using tools as gross as a Kjeldahl flask and a study of short duration. Nassett (1957) has recently published an evaluation of the findings of studies of amino acid requirements of rats and of men and women, and reports a similarity in the requirements of these groups when the requirement was expressed as milligrams per  $\text{kg}^{.75}$ .

## IV CONCLUSION

### A MINIMUM REQUIREMENTS

The tentative minimum requirements for the essential amino acids are summarized in Table X for women and for men (Rose, 1949). The requirements suggested for women are somewhat lower than those given for men—both on the basis of total daily amount and per kilogram. However, there were definite differences between the studies in the diet and technique used and the interpretation of the figures secured. These account for some of the differences in the results, and differences in the use of the results in stating requirements. In general the suggestions made for the requirements of women, especially for threonine, valine, tryptophan, phenylalanine with tyrosine and leucine, tend to be conservative based on means of a group of subjects and a zone of equilibrium, whereas those for men were based on the highest amounts needed by any subject for a slight but distinctly positive nitrogen balance. No suggestions are made for the "safe intakes" for women as Rose has made for men by doubling the amount of the minimum requirements.

### B AMINO ACIDS IN SELF CHOSEN DIETS

Also given in Table X are figures for the amino acid content of ordinary and self chosen food intakes as reported by Reynolds *et al* (1953), Futrell *et al* (1952), Mertz *et al* (1952) and Wharton *et al* (1953). These figures indicate that the foods available to people in this country are likely to supply ample amounts of the essential amino acids.

### C FURTHER NEEDS IN RESEARCH

Even with these indications of amino acid requirements and intakes, our information is far from complete. Much more needs to be known about the interrelationships among the amino acids and between the amino acids and other dietary items, of the rates of synthesis of amino acids by the body and of the value of the so called dispensable amino acids in protein nutrition and nitrogen balance. Much more work also needs to be done with subjects similar to the ones studied here and with subjects in different age groups. Individual variation in requirement among the subjects studied has been considerable. Some of this variation could probably be avoided with more rigid control in selecting and managing the subjects and with more attention to nutritional history or background and stage of physiological maturity of the subjects. However, the more homogeneous a group of subjects the less representative it may be of the larger population group one is trying to study.

The chief use of figures for the quantitative requirements for the

TABLE X  
COMPARISON OF TENTATIVE MINIMUM REQUIREMENTS OF AMINO ACIDS WITH AMINO ACID CONTENT OF AMERICAN DIETS

TABLE X COMPARISON OF TENTATIVE MINIMUM REQUIREMENTS OF AMINO ACIDS WITH AMINO ACID CONTENT OF FOOD INTAKES						
	Minimum requirements <sup>a</sup>		Amino acid content of food intakes			Wharton <sup>e</sup>
	Women	Men (gm./day)	Reynolds <sup>b</sup>	Futrell <sup>c</sup> (gm./day)		
				Mertz <sup>d</sup>		
Amino acid	0.45	0.70				28-31
Isoleucine	0.62	1.10			0.7-4.5	44-49
Leucine	0.50	0.80			13-78	35-40
Lysine	} 0.55	1.10	4.2	249-573		
Methionine			6.5	328-735		
Cystine	0.22		4.0	17-86		0.9-1.0
Phenylalanine	0.90			0.90-2.54	0.7-2.7	2.5-2.7
Tyrosine	0.31	0.50	3.0	1.98-4.88	0.9-3.77	
Threonine	0.16	0.25	4.1			2.6-2.9
Tryptophan	0.65	0.80			0.9-3.8	0.4-0.5
Valine					—	3.1-3.4
					0.8-1.9	
Values for men are from Rose (1949)						
Values reported in this chapter						
for women are from the studies reported in this chapter						

Values for men are from Rose (1949)

Values for women are from the studies reported in this chapter

<sup>a</sup> Values for women are from (1953)

<sup>b</sup> Reynolds *et al* (1952)

<sup>c</sup> Futrell *et al* (1952)

<sup>d</sup> Mertz *et al* (1952)

<sup>e</sup> Wharton *et al* (1953)

amino acids is in helping to identify possible limiting factors in human diets especially those diets in which plants are the major source of protein. Knowledge of such limiting factors and of the amino acid composition of indigenous foods will provide the basis for planning how best to supplement the plant proteins within the framework and economy of the existing food patterns of different population groups.

Our knowledge of amino acid requirements is still meager, still small in relation to what we need to and want to know about the subject. To date we have only a few road signs on the highway leading to more facts and better understanding. The road signs have been supplied by researchers with somewhat different approaches to the problem and different interpretations of their results. One of their contributions has been to give others a place to start from in conducting further research in this area. No longer need we feel if I were going there I wouldn't start from here." We have several starting places and there are many directions we can travel toward an increased knowledge of all aspects of amino acid nutrition.

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## CHAPTER 16

# Nutritional Needs of the Aged

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## I INTRODUCTION

During the past twenty years there has been a significant increase of interest in the nutritional problems of the older age groups. This interest has been manifested by basic research scientists, physicians, dietitians, governmental and social agencies of various types, food technologists, and many other groups, including the general public, as reflected in the lay press. This increased interest has been sparked by the certainty of an ever increasing number and percentage of citizens in the age groups above 50 years. According to the U. N. Secretariat (1956), approximately 5% of the population of the United States in 1900 was over 64 years of age, and this percentage doubled in the succeeding fifty years. Furthermore, due to a marked increase in the rate of population growth, it can be estimated that by 1975 there will be 20 to 25% of the world population over 64 years of age.

The United States Department of Agriculture (1955) reported a nation-wide household food consumption survey in which the data were collected by personal interview with the homemakers. They found that probably not more than 10% of the nation's household diets could be called "poor" and that women above the age of 55 years, when living

alone, showed a nutritional intake approximating that of a normal household

Albanese (1956-1957) pointed out that national food habits frequently result from rules and regulations which were originally imposed because of environmental or religious factors, many of which have long since been forgotten. Most societies eat the foods which are harvested locally and various limitations of food intake grow worse in the more underdeveloped portions of the world.

Increasing world population has already reduced the acres under cultivation to 17 per individual (Albanese, 1950a) whereas the experts maintain that 25 acres per individual are needed for complete sustenance. Argentina, Australia, Canada, and the United States are the only population groups that enjoy an excess of cultivated acres per inhabitant. It is interesting to note that the average height of the individual is definitely greater in those countries which enjoy a more optimal diet and that second generation Japanese born in California, are larger and enjoy better physique than their first generation American Japanese parents. The most important apparent change in diet habits within the United States during the past fifty years has been an increased consumption of proteins of animal origin at the expense of cereal products and potatoes. This change has resulted in an increased intake of protein foods of high biological value.

The results of long range observations of the growth rate as evidenced by college freshmen, were published by Chenoweth (1937). He reported that first year male students at the University of Cincinnati had shown an average increase of 27 inches in height and 224 pounds in weight, and the women entering college had added 13 inches in height and 43 pounds in weight—as compared to entering freshmen thirty-three years ago. He attributed the increased growth to a higher standard of living, increased knowledge of nutrition, and lower incidence of communicable disease.

As a result of these demographic developments the scientific literature pertaining to nutritional problems of the aging has grown by leaps and bounds making it almost impossible to review it completely in the space available here. We will attempt, therefore, to cover only some of the more pertinent research in this field with special emphasis upon problems. We shall also be concerned with the nutritional factors involved in the maintenance, repair, and prevention or retardation of the disintegration process which accompanies increasing age. It should be mentioned that during and following periods of stress the older may develop, temporarily, nutritional needs more or less in common with those of the growth period. It has long been known for instance that there is a

difference in the requirements of the oldster during different nutritional states (McCollum and Steenbock, 1912) Although much has been done in an effort to determine the factors involved in the difference in requirements for the older age groups more research will be needed before satisfactory knowledge in this area can be developed

## II PHYSIOLOGY AS RELATED TO AGING IN MAN AND ANIMALS

### A TISSUE CHANGES

About twenty years ago Simms and Stolman (1937) carried out chemical analyses of certain tissues in people over 70 years of age who had died from accidents. Similar studies were done on tissues of 11 persons between 30 and 40 years of age who had also suffered accidental death. Results showed that aging is accompanied by an increase in the chloride sodium calcium and total base and by a decrease in the potassium magnesium phosphorus nitrogen and ash content. Among these victims of accidental death the water content of the tissues was found to be greater in the older age group than in the younger. Another group of individuals 65 to 70 years of age were studied following pathological death and did not show the increase in tissue water.

Some years later Brozek and Keys (1950) pointed out the fact that changes in body composition which accompany the process of aging result in fatty tissues replacing some of the normally active tissues. This leads to an inaccuracy in the standard height weight tables and the phrase "per kilogram of body weight." Brozek (1952) further reported supplementary observations of 25 subjects 23 to 29 years of age 44 subjects 48 to 52 years of age and 34 subjects 53 to 57 years of age. The mean figures for these three groups are shown in Table I.

TABLE I  
CHANGES OF BODY COMPOSITION WITH INCREASING AGE<sup>a</sup>

Body composition	23-29 Years	48-52 Years	53-57 Years
Age	25.2	50.0	54.6
Height (cm)	176.5	175.4	174.8
Gross body wt (kg)	70.6	75.9	76.0
Specific gravity (Submersion)	1.0695	1.0500	1.0475
Per cent fat	14.4	24.0	25.2
Fat free weight (kg)	60.4	57.7	56.8
Skinfolds (mm)			
Abdomen	19.1	26.0	26.0
Back	14.6	20.4	21.5
Thigh	8.9	9.5	9.4
Chin	8.9	10.8	11.0

<sup>a</sup> Adapted from Brozek (1952)

Albanese (1953) summarized the current opinions of his group and of other workers regarding nutritional physiology in oldsters. His observations were made on a series of 219 female residents of a very high caliber Home, whose ages ranged from 66 to 94 years. He felt that the collected data indicated that the water content of the body decreases with age, suggesting that the daily requirement for water to meet normal physiological demands is about 50% less for the oldster than for the younger age groups. An excess of water intake in the oldster serves only to increase the cardiac load and kidney stress, and may result in the "washing out" of some important water soluble nutrients. Albanese found that the decrease in water content of the oldster is accompanied by a percentage increase of body fat, which suggests the possibility that the increased fat intake desired by many oldsters may have some physiological basis, in terms of Richters' (1941, 1943) studies on the self selection diet of rats. With regard to the protein intake in old age, Albanese believed that the decrease in lean body mass makes it unlikely that protein storage can be considerably increased by means of increasing the protein intake. On the other hand, an excessive protein intake is known to increase the blood urea content.

Torre *et al* (1955) reported investigations, by chromatographic techniques, regarding 13 amino acids found in the spinal fluid of normal oldsters, as compared with normal young subjects. The only divergences he noted were an increased glutamic acid level in the senile group and the fact that aspartic acid was the only free amino acid found in the oldster which did not appear in the spinal fluid of normal young subjects.

## B BASAL METABOLISM

In a group of subjects between 90 and 130 years of age, Turovets (1938-1939) found that the basal metabolism was 50% of the accepted normal for younger adults. The minute volume and systolic volume of the heart were also lower, and there was a greater difference between the oxygen content of arterial and of venous blood in the oldster. Kountz and his associates (1949) in a study of 78 men and 63 women varying in age from 25 to 100 years, substantiated this reduction in BMR with advancing age and found that the mean value for the serum organic iodine also falls with aging. No correlation between serum iodine and blood cholesterol levels was observed.

Brozek (1952) concluded from his investigations that the average caloric intake during advancing maturity tends to be larger than the actual caloric need, resulting in an increase in the deposit of fat. Although such fat serves as a fuel reserve, it can hardly be regarded as biologically desirable in spite of the fact that increased fat content of the body is a

"normal" accomplishment of aging in a statistical sense. He felt that the recommended daily dietary allowances of the Food and Nutrition Board of the National Research Council (1948) should provide an adjustment for age as well as for sex and activity. Using the age of 25 years as a base, he suggested a reduction of 7.5% in the recommended daily allowances for each decade above 25 years of age. [The Food and Nutrition Board did subsequently provide an adjustment for age in their recommended daily allowances (National Research Council Food and Nutrition Board 1953-1958).]

Binet and Bourhere (1951) felt that the previous studies of BMR in the aged were handicapped by the small number of observations and by the difficulties experienced in attempting to control and measure the dietary intake. Their work showed that in males between 70 and 80 years of age the BMR was 34.9 calories per square meter per hour and fell after 80 years of age to 30.8 calories per square meter per hour. Females between 70 and 80 years of age showed a BMR of 34.4; those above 80 years 32.9; and those above 90 years 32.5 calories per square meter per hour—representing a gradual decrease with advancing years. This substantiated the results reported by Kountz and his co-workers (1949). It was further noted by these investigators that of their subjects 34 hypertensives showed a BMR above the average for their age group.

Studies reported by Albanese (1953) suggested that when oxygen consumption is expressed in terms of the active tissues (lean body mass), the observed decrease in the standard BMR with aging is less marked. Shock and his associates (1955; Shock and Yiengst 1955) carried out studies in 170 ambulatory males varying from 19 to 91 years of age with regard to oxygen uptake, carbon dioxide elimination, total respiratory volume (measured under basal conditions by the Tissot open circuit method), the interrelation between basal oxygen consumption and thiocyanate space, antipyrine space, inulin and PAH clearance. They felt that their results demonstrated that although BMR calculated per unit of surface area decreases with age, the oxygen consumption per unit of total body water or per unit of intracellular fluid shows very little change with advancing years as a result of a parallel decrease in oxygen uptake and in body fluid with age.

### C ORGAN FUNCTION

In studies of liver function and gastric secretory response, Rafsky and Newman (1943-1947) found that 26% of 100 subjects above 60 years of age showed abnormal findings in 4 or more liver function tests, but only 2% of the subjects showed decreased liver function in 3 tests. Following a test meal of bread and water, 53% of the subjects showed 19 units or

less of free hydrochloric acid in the gastric juice, none showed complete achlorhydria, and 13% showed some degree of hyperchlorhydria.

Olbrich and his co-workers (1950) compared renal function of subjects over 60 years of age with that of normal young men. They measured the inulin clearance, diiodone clearance, and tubular excretory capacity for diiodone, and found a reduction in kidney function in the oldest of 25 to 30% for those with normal diastolic blood pressure, and 35 to 50% in subjects with elevated diastolic pressure. These investigators felt that this did not signify impairment of the glomerular filtration, but rather reflected changes in the renal plasma flow accompanying the vascular changes indicated by the diastolic blood pressures.

The effect of a protein free diet for 30 days upon the activity of liver enzymes in adult rats of various ages was reported by Ross and Ely (1951), who found that the enzyme activity of the adult protein depleted rat was similar to the activity in the young rat.

Horwitt (1953) studied the absorption of nutrients from the gastrointestinal tract in a group of men more than 70 years of age, as compared with a group about 30 years of age. Both groups showed equal absorption of glucose, thiamine, and riboflavin (after a period of restricted intake) and nitrogen when on normal diets supplying 65 gm of nitrogen per day. When there was a deficient vitamin intake, however, the older men showed biochemical differences with higher levels of blood lactic and pyruvic acids.

#### D LONGEVITY

Sherman (1941) reported that rats fed a diet proven to be adequate through 50 generations showed a deferment of old age when the intake of proteins, calcium, riboflavin, and vitamin A were increased. On the other hand, McCay and his associates (1935, 1938, McCay, 1941, 1942) carried out 5 series of feeding experiments using rats. At middle life, the diets were identical except for the amount and quality of protein or the caloric value. When the percentage of protein was the only variable, there was no significant change in the life span. They concluded from their results that the important factors which influenced the life span were those associated with the degree of fitness of the body. Irrespective of the amount of exercise taken, the rats which were kept underweight by caloric restriction outlived those which became fat on a full diet. It was also observed that at high levels of protein intake the blood nitrogen was increased and the heart and kidneys became larger.

These findings of McCay's group were supported by Ball and his co-workers (1947) in studies of weaned mice. Two groups of mice were fed the same amount of protein, vitamins, and minerals, but the experimental

group received less carbohydrate and fat, resulting in a caloric intake about 30% lower than that of the control group. All of the mice were kept on the regimen for 240 days after weaning and the experimental group was then returned to *ad libitum* intake of the normal diet. The mice on a restricted caloric intake showed a definite delay in maturation, with a full recovery after return to a full diet. The longevity of this underfed group was greater than that of their controls, with 25% still alive when all of the fully fed litter mates had died. McCollum and his associates (1939) felt that the diets used in McCay's experiments were not entirely suitable for the purpose. They pointed out that although food restriction did promote longevity statistically, the lengthened life span was accompanied by a decided lack of well being in the diet restricted animals. (Authors note: In other words the animals enjoying increased longevity added years to their lives rather than life to their years.)

### III PROTEIN NUTRITION IN OLD AGE

#### A REQUIREMENTS AND INTAKE

Albanese (1947) discussed the then current knowledge and beliefs regarding protein nutrition in the aged. He admitted that little was known of the biochemistry of the aging process and mentioned that Robertson (1908) had discussed the metabolism of old age and that few advances had been made in the subsequent forty years. Fenger (1904) had reported his observations of a 61 year old woman weighing 42 to 45 kg. over a period of fifteen years on a diet which furnished about 2 gm. of protein and 25 to 30 calories per kilogram per day. He noted no serious impairment in this woman's ability to utilize food satisfactorily with her advancing age. Albanese also referred to protein balance studies by Koch (1911) in 5 men aged 54 to 79 years. These men received diets of meat, potatoes and vegetables providing an average of 10.6 gm. of protein nitrogen daily; they utilized an average of 86% of the protein. Advancing age caused no appreciable diminution of this ability. Albanese concluded that from available data it was impossible to establish the definite protein requirements for the different physiological states of man and recommended that generous allowances such as those advocated by the Food and Nutrition Board of the National Research Council (1945) be used, i.e. 35 gm. of protein per kilogram per day up to 1 year of age, then gradually decreasing to 1 gm. per kilogram per day for adult men. Albanese believed that the quantity of protein needed is greatly influenced by the protein quality and that knowledge of these matters was still in the frontier stage.

Kountz (1947) reported diet experiments in 27 oldsters who were fed 2000 calories daily with a caloric distribution of protein 17%, carbo



hydrate, 47%, and fat, 36%. Since 41% of the subjects showed negative nitrogen balance in spite of this ample intake, he felt that high protein feeding was indicated especially following periods of protein depletion and that protein accumulation in the body would result in a positive nitrogen balance.

Meyer (1947) referred to earlier studies (Meyer, 1940) which indicated a decrease in the volume, acidity, and pepsin content of the gastric juice with advancing age. Trypsin concentration in the pancreatic secretion was not affected. His clinical experience led to the conclusion that the average elderly person digests protein well in spite of the aging process.

Kountz and his co workers (1948) supplemented their previous work (Kountz, 1947) by studies which indicated that a positive nitrogen balance could be produced in the elderly, and maintained for at least 3 days by a dietary intake of 2 gm of protein per kilogram per day. This suggests protein storage to compensate for a deficit engendered by a previous faulty diet.

In similar investigations (Roberts, 1948), 9 normal active women from 52 to 74 years of age were allowed, for at least 4 months, a self selected diet which was modified from time to time by changes in milk, vitamins, and calcium. The average intake of nitrogen was 8.9 gm per day, with a range from 6.8 to 11.4 gm. The results indicated that nitrogen equilibrium was maintained in 87% of these women. A group of 11 men who had not consumed meat, poultry, or fish for 12 to 47 years (Mirone, 1950), averaged 5.7 gm of animal protein daily, derived from skim milk, cheese, butter, and eggs. The total protein intake averaged about 50 gm per day. Since these subjects showed no demonstrable impairment in general health, Mirone concluded that the recommended daily allowances of the National Research Council (1948)—70 gm of protein per day, with 50% of animal origin—were too high, and that vegetable protein combinations could be satisfactorily substituted for animal protein.

Further nitrogen balance studies in elderly men 69 to 76 years of age were reported by Kountz and his associates (1951). Their subjects were fed 0.5 gm of protein per kilogram per day for 60 days, 0.34 gm for 15 days, and 0.7 gm for 110 days. Diets containing less than 0.7 gm of protein per kilogram per day produced negative nitrogen balance in all subjects, with a loss of body weight in 75% of the subjects. Albanese and his co workers (1952a) compared their findings regarding the daily food intake of healthy women 66 to 94 years of age, with results obtained in a similar group of women by Ohlson *et al* (1950). A group of women of similar age but chronically ill, as reported by Vinther Paulsen (1950), and with the recommended daily allowances

of the Food and Nutrition Board, National Research Council (1948) for sedentary adults. It will be noted in Table II that none of the three groups of investigators found as high a caloric and protein intake as those recommended by the National Research Council. Albanese's group felt that normal women above 65 years of age can maintain a state of good health with normal blood proteins on a lower food intake than is customarily advised.

TABLE II  
COMPARISON OF ACTUAL FOOD INTAKE OF AGING WOMEN WITH NATIONAL RESEARCH COUNCIL RECOMMENDATIONS

Source	Age range	Calories	Protein (gm)	Status
National Research Council (1948)	Adult	2100	60	Normal Sedentary
Ohlson <i>et al</i> (1950)	70-77	1500	53	Normal
Vanther Paulsen (1950)	66-85	1037	29	Chronically ill
Albanese <i>et al</i> (1952a)	66-94	1895 $\pm$ 102	43 $\pm$ 5	Normal

About this same time Keys (1952) reported that inasmuch as the body size and metabolic rate decrease with advancing age (thereby requiring about 5% fewer calories per day for each decade after 50 years of age) he felt oldsters can maintain their body weight on an intake of approximately 1500 to 2000 calories per day and that the protein intake of the oldster should remain at about 1 gm per kilogram per day as recommended for the middle aged group. He emphasized that the dietary proteins should come from a variety of sources to assure a proper balanced intake of nutrients.

A qualifying doubt regarding the previously reported results of nitrogen balance studies was expressed by Hegsted (1952). He pointed out that an individual on self selected diet would show a nitrogen balance varying from a positive balance through equilibrium to a negative balance depending upon the changing factors of stress or food intake. Horwitt (1953) reported studies along the same lines as those of Kountz (1947) and Hegsted (1952). Thirty one individuals who had been on a diet yielding 11 gm of nitrogen per day were found to be in positive nitrogen balance. When their diet was changed so as to reduce the nitrogen intake to 6.5 gm per day they immediately went into negative balance. With the continuation of the same intake however over a period of 3 months these individuals went back to a positive nitrogen balance. There was no difference noted between the oldster and the young adult in this respect. He agreed with other workers that these data cast a doubt upon the nitrogen balance technique of determining protein requirements.

Studies by Kountz and his associates (1953) extending earlier work (Kountz *et al.*, 1951), concerned the effect of varying levels of protein intake in oldsters as reflected in nitrogen balance, glucose tolerance blood proteins, blood NPN, and metabolic rate. The level of protein intake varied from 0.7 gm per kilogram per day, through various levels, to a maximum of 2.5 gm per kilogram per day. The protein used was mostly of animal origin. The only significant differences noted were a feeling of satiation with poor appetite and a rise in plasma NPN, at the high levels of protein intake. In the opinion of Albanese (1953), a daily protein intake of 0.6 gm per kilogram is sufficient for the requirements of the normal oldster provided that the proteins are of high biological value with 30 to 50% of animal origin.

Other studies regarding the protein metabolism of oldsters were reported by Schulze (1954, 1955). He found that the minimal endogenous nitrogen excretion is lower in the aged and parallels the decreasing metabolic rate and that proteins of animal origin are more useful than those of vegetable origin. His nitrogen balance experiments indicated that a protein intake of 0.5 gm per kilogram per day maintains equilibrium in the oldster as well as in the young adult provided the total caloric intake is adequate. A group of 36 hospitalized healthy volunteers between the ages of 60 and 92 years were fed a high caloric, high carbohydrate diet and the protein metabolism was estimated after a 10 day period. The results suggested that the minimum amount of protein required for nitrogen balance in the aged is equal to that required in the healthy young adult—roughly 0.5 gm per kilogram per day. This requirement should be increased by a safety margin to overcome the effects of stress. Schulze felt that the oldster weighing 60 kg could take 1.0 to 1.5 gm of protein per kilogram per day in a mixed diet of about 2000 calories per day. (This work follows the same lines and states the same opinions as those reported by other workers above.)

### B PROTEIN AND AMINO ACID METABOLISM

In recent years, many investigators have interested themselves in the protein and amino acid metabolism of the oldster as compared with the young adult. Bock (1947, 1948) measured the total serum protein in elderly men and women under 80 years of age and men and women over 80 years of age. The findings indicated a slight trend toward a lowering of the total serum proteins with advancing age. The elderly male, over 80 years, tends to show a slightly higher total serum protein than the female. The A/G ratio likewise falls with advancing age largely at the expense of the albumin fraction. (All of the changes reported were of relatively slight degree and may not be statistically significant.) The

results of similar studies reported by Olbrich (1948) in a group of 78 men and women 60 years of age or over substantiated the findings of Bock (1947-1948) with regard to the trend of the total serum proteins and the A/G ratio with advancing age. In addition there was a gradual increase in blood NPN and an increase in the erythrocyte sedimentation rate in the older group which Olbrich assigned to the change in the plasma proteins. Rofsky and his associates (1949, 1952), Manzoni *et al* (1952) and Vinzetti and his co-workers (1952) all supported the findings of Bock (1947-1948) with regard to the plasma proteins and globulin. Of 21 men and women between 70 and 95 years of age 76% showed a serum globulin content of 33% or more of the total circulating protein, the  $\beta$  globulin fraction was higher in the older than in the young adult and the polysaccharide content also increased.

The storage of protein in 4 healthy males and 6 undernourished males was studied by Levey and his group (1949). The plasma volume (dye method), total circulating proteins and the albumin fraction were measured before the intravenous administration of 1000 ml of physiological saline solution and determined again 30 minutes after the injection. The 4 normal subjects showed a constant response to the saline infusion with an increased plasma volume and an increase in the total circulating protein but a decrease in serum protein percentage concentration suggesting a "washing" of proteins into the circulation from labile stores. The 6 malnourished subjects responded by a decrease in both the total circulating proteins and the plasma protein percentage concentration in spite of an increase in plasma volume suggesting their inability to mobilize sufficient protein from the deficient protein stores. When these undernourished subjects were then placed on a high calorie high protein diet (4000 to 5000 calories per day) for a period of 3 weeks they showed a response to the saline infusion similar to that found in the normal healthy group.

Mitchell (1950) studied the utilization of nitrogen from 6 test proteins (expressed as absorbed nitrogen per calorie of heat needed for nitrogen equilibrium) in adult rats and adult humans. The results were similar with the exception of the rats increased special requirement for cystine methionine used in the production of keratin. This indicated to him that the total amino acid needs of all mammals are dependent upon the pattern of essential amino acids in the particular protein tissue being formed or catabolized at a given time.

Silber and Porter (1950) fed a protein free diet to rats for 1 week and then fed Vuj amino acid mixture (Madden and Clay 1945) at a level of 300 mg nitrogen per kilogram per day. An excess load (100 mg of nitrogen per kilogram) of the single amino acid to be tested was then

added for 1 day and the per cent of the excess amino acid nitrogen excreted was measured. They tested 7 essential and 4 nonessential amino acids individually, and the results showed an imbalance effect for methionine. For each milligram of excess methionine nitrogen fed the animals excreted 1.86 mg of nitrogen in the urine. In contrast only the excess dietary tryptophan, lysine, and leucine nitrogen were lost in the urine, with no imbalance effect. Feeding an excess of excess nitrogen was partially utilized.

Further animal studies were reported by Chow (1950) and indicated that any amino acid should be considered a dietary essential if the animal cannot synthesize that particular amino acid at a rate commensurate with the body's need for that particular amino acid at that particular time. Synthesis of a complex protein molecule requires the presence of all the necessary essential amino acids at the proper time and in the proper amounts. This is illustrated in the protein-depleted dog that responds to the feeding of casein hydrolyzate by an increased production of both albumin and globulin, whereas the feeding of lactalbumin favors the regeneration of blood albumin. Chow felt that the possibility of a directive substance for the synthesis of body proteins must be further studied.

Plasma levels of free amino acids in the elderly and young adult groups were compared by Hofstatter and his group (1950), using microbiological techniques. The oldsters showed significantly lower plasma levels of valine, tryptophan, lysine, leucine, and isoleucine, and a higher value for histidine, when compared with the young adults. There was no significant difference noted for threonine, tyrosine, and glycine.

Steghitz (1950) offered the following formula for determining the degree of protein deficiency at a given time. The plasma volume equals 5% of the total body weight, and the normal plasma protein is about 5 gm per 100 ml. Therefore in a patient weighing 70 kg, the blood plasma weighs 3.5 kg or is approximately 3500 ml in volume. If this individual showed a total plasma protein of 5 gm per 100 ml, there would be a deficiency of 2 gm per 100 ml which when multiplied by 35, indicates a deficit of 70 gm in the total plasma protein. It is estimated that there exists a 30 gm depletion in the tissue protein reserves for each gram of deficiency in the plasma protein. Therefore,  $30 \times 70 = 2100$  gm depletion of the tissue protein, and the total protein deficit of the individual is 2170 gm. To correct this deficit it would be necessary to add to the normal protein intake of 1 gm per kilogram per day, an additional 2000 gm (2 kg) of protein distributed over the rehabilitation period. During

this period the individual would be in a positive nitrogen balance continuously until the deficit is made up

Measurements of the specific dynamic action of proteins in young and old subjects were reported by Horvath and Tuttle (1951). They fed a test meal of egg whites and ground beef yielding 25 gm of protein to one group of males with a mean age of 77 years and to a second group of males with a mean age of 24 years. Each test meal was analyzed for nitrogen content. The utilization of oxygen was determined prior to giving the meal, and again every 30 minutes, for 5 hours postprandial. The results indicated that the older showed more variation in the specific dynamic action but that the mean response tended to be greater.

Mertz and his co-workers (1952) by means of microbiological analyses of food components collected during nitrogen balance studies in 18 old women on self-selected diet determined the daily intake of 7 essential amino acids. The intake of isoleucine, leucine, lysine, threonine, and valine met or exceeded the phenylalanine intake was intermediate, and the methionine intake rarely reached—the levels reported by Rose (1949) for young adult males.

The effects of amino acid deficiencies in adult Norwegian rats were reported by Albanese (1952a). One group of animals received a diet containing only 0.43% nitrogen derived mainly from yeast while the control group diet yielded 2.51% nitrogen. In the low nitrogen intake group the rats lost weight, blood protein levels dropped 20%, and the A/G ratio decreased at the expense of the albumin. Analysis of the tissues indicated that the protein deprivation effect was of different magnitude in various organs with the liver suffering the most. In another experiment a group of rats were fed a normal diet with the exception of a deficiency of a single amino acid at one time. Nine essential amino acids were tested in this manner as well as some of the nonessential group. The results suggested that a dietary deficiency in a single amino acid because of the imbalance caused more profound deleterious effects than did the low protein regimen.

In further studies employing human subjects of both sexes Albanese (1952a) fed weighed diets providing approximately 40 calories per kilogram per day with 0.1 gm of nitrogen per kilogram per day. Ninety per cent of the protein moiety consisted of a protein hydrolyzate which was deficient in some one amino acid. Thirty such experiments with 7 amino acid deficiencies were carried out. Each experimental period was preceded and followed by a control period in the same subject during which the amino acid in question was present in the diet. The nitrogen balance data showed that tryptophan, lysine, and methionine were essential parts of the diet for the maintenance of nitrogen equilibrium whereas histidine

and arginine were not. He referred to the work of Rose (1949) which demonstrated the essential character of other amino acids as mentioned above. In attempting to identify the different amino acid deficiency states in terms of blood protein or urinary constituents, Albanese found poor correlation between the amino acid intake level and the blood hemoglobin, albumin, globulin, and A/G ratio. Analysis of the urine, however, in a subject receiving a tryptophan deficient diet showed a sharp decrease in the excretion of tryptophan which could be returned to the normal range by the addition of 30 to 60 mg of tryptophan per kilogram of body weight. The development of chromatographic analysis simplifies this approach to the study of amino acid metabolism and should be of considerable value in future investigations.

Horwitt and co workers (1953; Horwitt, 1953) studied the amino acid metabolism of 11 old men and 9 young men, over a 3 year period. The intake and excretion of the essential amino acids were measured while the subjects were on a basic diet furnishing less than 60 gm of protein per day, and less than 50% of the protein derived from animal sources. It was found that less than 1% of the ingested amino acids were excreted, and the older group showed a slightly higher amino acid excretion.

A study of the quantitative dietary need for the essential amino acids, by men over 50 years of age, required to maintain nitrogen equilibrium was reported by Tuttle *et al* (1957). Previous investigations into the needs of the younger age groups indicated that at least 8 amino acids were essential in the diet to maintain nitrogen balance. Rose (1949) gave minimal intake figures as shown in Table III. Holt *et al* (1941), Leverton *et al* (1956a, b, c, d, e), Jones *et al* (1955), Harte and Travers (1947), and others had reported studies of the requirements of young adults for certain single amino acids. Tuttle's group concerned them

TABLE III  
INDISPENSABLE AMINO ACID NEEDS OF MEN OVER 50 YEARS OF AGE

Amino acid	Test mixture (gm)	Rose minimum (gm)	Tests $\pm$ %
l Leucine	1.72	1.10	+56
l Isoleucine	1.28	0.70	+83
l Lysine	1.39	0.80	+73
l Threonine	0.77	0.50	+54
l Tryptophan	0.38	0.25	+52
l Valine	1.29	0.80	+61
l Methionine	0.56	1.10	
l Cystine	0.45	spares methionine	
l Phenylalanine	0.96	1.10	
l Tyrosine	0.60	spares phenylalanine	
l Histidine	0.43	not essential	

selves with 5 healthy men in the age range 52 to 68 years. They were admitted to a metabolism ward for the study period, and were allowed to ambulate and work at hobbies etc. They were examined by a physician daily, and all necessary laboratory tests performed to assure a state of "good health." The control period diet was composed of natural foods furnishing about 7 gm of nitrogen per day from good quality protein, and a caloric intake of 2014 to 2717 per day. When nitrogen equilibrium had been established by carefully performed nitrogen balance techniques the protein of the control diet was replaced by the test mixture of amino acids shown in Table III, plus enough glycine to bring the total nitrogen intake up to about 7 gm per day. The ratios of the amino acids in the test mixture were designed to approximate the composition of 1875 gm of egg protein. An adequate mineral and vitamin supplement was also given daily. Figure 1 shows the nitrogen balance data during seven diet periods in one individual which are representative of the group. It can be seen that a mixture of essential amino acids as described in Table III, plus a small amount of additional tryptophan and enough glycine to raise the total nitrogen intake to about 7 gm per day, was not sufficient to meet the nutritional requirements of the older men and resulted in a negative nitrogen balance. This also occurs with the feeding of 1875 gm of egg protein plus glycine to bring the total nitrogen intake up to about 7 gm per day to match the control diet. When the amounts of essential amino acids in the basic mixture were doubled however nitrogen equilibrium was achieved. The authors remark that although the addition of small amounts of nonessential food nitrogen to the diet has been shown to exert a sparing effect upon the essential amino acids recent studies seem to indicate that a high glycine intake may actually increase the dietary requirement for essential nitrogen in the human.

According to Sharpenak (1957) the optimal composition of amino acids in human food is that which is close to the average composition of the amino acids in the proteins of the human body. Red muscles of the human body contain the following percentages of amino acids (based on nitrogen content) as per cent of total protein nitrogen of the muscle—valine 4.0 leucine 6.5 arginine 13.4 histidine 4.1 lysine 9.7 tyrosine 1.9 phenylalanine 2.4 tryptophan 1.9 cystine 1.3 and methionine 1.7. On a weight basis these amino acids constitute correspondingly 6.0 9.9 6.7 2.4 8.1 4.0 4.7 2.2 1.8 and 2.8% of the muscle proteins.

Data are tabulated representing the average amounts of these amino acids in the various organs of 25-35 year old men with an average body weight of 70 kg. The amounts of the individual amino acids vary from organ to organ. However the average per cent composition of the



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a period of 8-17 days. At a later date the same men received an isocaloric diet containing small amounts of egg yolk which increased the amount of protein nitrogen in the diet to 2.191-3.294 gm per day. The nitrogen balance was determined within the last 8-21 days of the diet intake and indicated that the daily excretion of nitrogen in the urine (1.382-2.134 gm) and feces (0.734-0.961 gm) was the same for both diets. The excreted nitrogen represents the so called endogenous loss of protein. Since this "endogenous" loss of protein was constant, it was possible to determine the nutritional value of milk and pea proteins in comparison to egg yolk. The biological value of these food proteins for human nutrition decreased in the order egg yolk, milk, and peas.

The fact that there is often no reliable correlation between the nutritional state of an individual and the blood protein concentrations was pointed out by Albanese (1952b) and by Albanese and Higgons (1953). Further studies showed that deficient dietary intake of tryptophan, methionine and isoleucine impaired the biosynthesis of plasma proteins whereas a deficient intake of lysine did not produce this effect. It appeared that arginine was substituted for the lysine to the extent of a 32% increase in the arginine content of the plasma proteins which returned again to the normal level when the lysine intake was increased to the optimal intake for a 3 week period. It is thought that this indicates a complete regeneration of the circulating plasma proteins in less than 21 days.

Results of blood protein studies in 130 men and 106 women from 60 to 90 years of age were reported by Herbeuval and his associates (1955). The albumin fraction was slightly decreased in more than half of the subjects and the  $\alpha_1$  and  $\alpha_2$  globulins were increased. Changes in the  $\beta$  and  $\gamma$  globulin fractions were less definitive with only one third showing a slight reduction, one third a slight augmentation and one third normal. Morgan and his group (1955) and Karel *et al* (1956) also noted a decrease in the A/G ratio in oldsters at the expense of the albumin fraction. There was no apparent correlation between the total plasma proteins and the dietary protein intake. The blood uric acid levels were not related to either the protein or the fat intake of the diet. Karel and his associates (1956) felt that the decreased albumin content in the oldster might indicate a diminished rate of albumin synthesis secondary to progressive decrease in the efficiency of the liver.

Chinn and his co workers (1956) studied protein metabolism in the aged from a new direction. They compared 12 aged persons 72 to 86 years of age with 6 younger subjects 16 to 61 years of age. Each group received a test meal containing iodine<sup>131</sup> labeled albumin and the radioactivity of the blood was determined at intervals up to 3 hours after the

amino acids in the total proteins of the human body is very close to the composition found for human muscle proteins. The entire body of a 70 kg man contains the following amounts of these amino acids: arginine 715, histidine 256, lysine 907, tyrosine 349, tryptophan, 167,

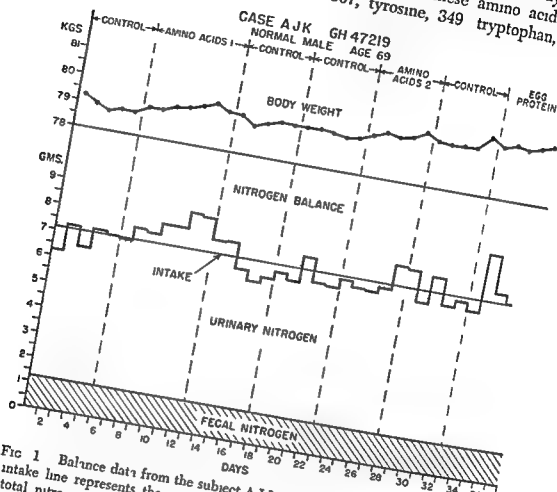


FIG 1 Balance data from the subject A J K. The irregular line above and below the intake line represents the sum of daily fecal and urinary nitrogen excretion or the total nitrogen output. The intake line is bordered by dotted lines equivalent to 0.13 gm which represents one standard deviation as observed in the analysis of the sample diets prepared for this subject. Amino acids 1 represents the basal essential amino acid mixture plus glycine and an additional 0.38 gm of tryptophan. Amino acids 2 represents double the basic amounts of essential amino acids. Egg protein represents 18.75 gm of natural egg protein with glycine added to bring the nitrogen level to 7 gm. From Tuttle *et al* (1957).

and cystine 171 gm respectively. The amino acid content of egg yolk proteins is very close to the amino acid composition of the human body. The nutritional values of several proteins were investigated in a study in which four 25-35 year old men were given a low nitrogen control diet providing from 0.200 to 0.243 gm of protein nitrogen daily for

amounts improved the utilization of the deficient proteins by the body. Albanese and his co-workers (1955b, 1957) indicated that the healthy oldster can maintain himself on a minimum daily protein intake of approximately 55 gm of which 30 to 40% is of animal origin if the diet affords 1600 or more total calories per day. During and following periods of stress, however, the same type of elderly patient was found to be in negative nitrogen balance in spite of a daily protein intake of 60 gm or more of which 30% was of animal origin. In such patients the supplementation of the diet by the addition of 600 to 900 mg of L lysine daily appeared to improve the utilization of the dietary protein and to reverse the negative nitrogen balance. Norris (1957) reported diet studies in 5 institutionalized women from 75 to 91 years of age who were offered a diet calculated to be adequate if sufficient quantity were eaten. The subjects, however, on a self-selected diet regimen tended to eat an excess of the starchy foods, thereby producing a poor diet balance. Supplementation of their diet with 600 mg of L lysine plus therapeutic amounts of vitamin B complex produced the following effects:

- (a) An increment of 0.8 to 3.1 gm % in hemoglobin in three months
- (b) A slight weight increase which was not considered significant
- (c) An increase in energy and improvement in mood
- (d) A decrease in the number of upper respiratory infections

Higsons and Albanese (1957) reported an extensive dietary experiment in which 415 convalescent adults with an average age of 65 years were studied. The subjects were divided into two groups. Group I of 114 test subjects and 190 controls received the regular institutional diet. The 114 test subjects received in addition a dietary supplement consisting of 50 gm of a milk protein concentrate (Somagen<sup>2</sup>) in 500 ml of whole milk. The mixture was divided into three portions and administered between meals and at bedtime for a period of 3 to 4 weeks. The daily supplement furnished 522 calories with a distribution of protein 41%, carbohydrate 28%, and fat 31%. The test subjects on this supplement gained weight at an average rate 1.4 times that of their controls.

In Group II, 47 test subjects and 64 controls also received the regular institutional diet. The 47 test subjects received in addition a daily dietary supplement consisting of 25 gm of the same milk protein concentrate (Somagen<sup>2</sup>) plus 25 ml of a mixture of essential fatty acids (Lipomul<sup>3</sup>) and 30 gm of sucrose in 500 ml of milk, furnishing a total of 652 calories with a distribution of protein 22%, carbohydrate 39%, and fat 39%. This supplement was divided into three portions as above and administered for 3+ weeks. The test patients in this group showed an average

<sup>2</sup> The Upjohn Company, Kalamazoo, Michigan

meal The total radioactivity of the feces was measured for 72 hours This technique showed no significant differences in the rate or the extent of protein digestion and absorption in the old versus the young group

### C EFFECTS OF VARIOUS SUPPLEMENTS ON PROTEIN METABOLISM

#### 1 Protein Supplements

For some years the use of protein supplements to increase the dietary protein intake of oldsters has been tried by various investigators Barrowsky (1940) reported the use of a fortified drink (Cocomalt) in 30 senile individuals with a history of questionable dietary intake due to various gastrointestinal disturbances The supplement was administered in milk, and each ounce of the combination afforded protein, 15 gm fat 14 gm carbohydrate 41 gm, and 36 calories The caloric distribution was protein 17% fat 36%, and carbohydrate 47%—which is approximately the ideal distribution for growth or repair Most of the subjects responded by an improvement in appetite, a gain in body weight and an increase in blood hemoglobin level

Some years later Pietra (1949) reported studies of 32 elderly patients suffering from various stages of fracture of the femur, with and without infection The aged bedridden oldster tended to show a negative protein balance even on a normally adequate diet An increase of the total protein intake to 100–150 gm per day by means of a protein supplement such as Protinal<sup>1</sup> (70% milk protein) was required to produce a positive protein balance

Improvement of protein nutrition by the addition of supplemental calories in the form of edible oil 50% sucrose 12%, emulsifying agent 2% and skim milk powder 23% was reported by Mindrum (1953) Nine patients who were suffering from protein depletion because of illness showed an anabolic effect of the supplement, with a reversal of the negative weight trend

According to Albanese (1955) it is generally conceded that proteins from animal origin are of high biological value with a good amino acid pattern The cereal and vegetable proteins tend to lack proper quantities of certain of the essential amino acids which are necessary for the synthesis of human tissues Experimental evidence showed that the dietary protein with an amino acid pattern resembling that of mammalian muscle tissue is utilized best by man (Mitchell 1950) Human muscle tissue contains approximately 6 units of lysine to each unit of tryptophan (L/T ratio 6:1) In the nonanimal proteins lysine is particularly apt to be deficient and supplementation of the diet with lysine in the proper

<sup>1</sup> National Drug Company Philadelphia Pennsylvania

TABLE IV  
NET CHANGES IN BODY WEIGHT AND SOME URINARY CONSTITUENTS OF PATIENTS RECEIVING TESTOSTERONE PROPIONATE ORALLY<sup>a</sup>

Changes in	Subject (age and sex)							Total for group
	WA 62 (m)	HJ 64 (m)	WS 68 (f)	JL 78 (f)	OD 49 (m)	HJ 62 (m)	JJ 56 (m)	
Weight (kg)	+ 0.5	0	+ 0.5	0	+ 1.5	+ 1.0	+ 3.0	
Blood constituents								
Total plasma proteins (gm %)	0	- 0.9	0	0	0	0	0	
Hemoglobin (gm %)	0	- 1.0	0	+ 1.2	+ 0.7	0	0	
Hematocrit (mm)	0	0	- 2.5	+ 6.0	+ 2.0	0	0	
Urinary constituents								
Total nitrogen (gm)	- 1.1	- 0.8	- 1.2	- 1.0	+ 3.7	- 1.0	+ 1.0	
Urea nitrogen (mg)	- 0.4	- 0.5	- 2.2	- 0.8	+ 2.0	- 0.9	0	
Amino nitrogen (mg)	+144	- 37	+ 8	+ 46	+187	+178	+163	
Arginine (mg)	- 46	+ 26	- 14	- 24	0	0	0	58
Cystine (mg)	0	+ 19	- 15	0	0	0	0	4
Histidine (mg)	- 48	0	- 45	- 12	+143	+ 67	+ 12	+ 117
Methionine (mg)	+134	+220	+139	+ 77	+266	+ 68	+281	+1165
Phenylalanine (mg)	+ 60	+ 69	- 55	+ 66	+197	-272	+333	+398
Tryptophan (mg)	+ 57	-104	- 34	+ 37	+357	+233	+162	+ 708
Tyrosine (mg)	+ 28	+150	- 30	0	- 81	- 63	- 27	- 23
Changes in amino acid excretion per subject (mg)	+185	+380	- 54	+144	+882	+ 33	+741	

<sup>a</sup> Dose of 10 mg daily

rate of gain which was 1.8 times that of their controls. Further in Group II, 37 of the test subjects, whose weight was less than 80% of standard expected, gained weight on the supplemented diet at a rate 2.0 times that of their 42 controls. The superior results in Group II were thought by these investigators to indicate the value of a properly balanced dietary supplement as contrasted to a mere increase in protein intake which is not protected by sufficient energy calories. It should also be noted that the nutritional status of the individual plays a definite part in the response to dietary supplements.

## 2 Hormone Supplements

Hormones which are indispensable are produced by the glands of internal secretion and are formed in the body from proteins and amino acids. They are divided into three classes: (a) amino acid derivatives, such as adrenaline and thyroxine, formed from tyrosine or phenylalanine; (b) protein hormones such as insulin, parathormone and ACTH which require nonessential as well as essential amino acids for their formation; and (c) steroids which come from the ovaries, corpus luteum, testes, and adrenal cortex—with which we are most concerned here. ACTH stimulates the adrenal cortex causing an increased output of the corticosteroids. Physiologically, these steroid hormones fall into two general categories: the group showing androgenic and anabolic activity, such as testosterone and its derivatives (Kochakian 1947) and the catabolic or catabolic group, such as cortisone and its derivatives (Fourman *et al.*, 1950).

Since both groups of substances are employed clinically in the aged it is appropriate to consider here some of their effects upon protein metabolism. The catabolic group affects the protein metabolism with resulting mobilization of amino acids from the tissue proteins for conversion to glucose and glycogen mobilization of fat stores for energy; increased excretion of nitrogen, potassium, and phosphate with the production of a negative nitrogen balance and an electrolyte imbalance with retention of sodium and chloride and disturbance of the water balance. The anabolic group of steroids affects the protein metabolism by promoting synthesis of protein tissues from the nitrogen containing foods.

Albansen and Higgons (1950) reported the results of studies aimed at clarifying the biochemical mechanism involved in the anabolic activity of the androgens in man. The data collected in Table IV summarize the effects of oral administration of 10 mg of testosterone propionate daily for 1 week upon the nitrogen metabolism as reflected in the urinary excretion of nitrogen metabolites, blood proteins, and weight changes in

the intermediary metabolism of proteins and result in a reduction in lean body mass. It was admitted however that the existing knowledge was fragmentary and that endocrine changes in old age might be the result rather than the cause of senescence, or might be contributed to by chronic undernutrition. It is essential that dietary proteins of high quality and other essential nutrients be continuously consumed in sufficient quantities for a sufficient period of time by the older under study, before we can implicate changes in the intermediary metabolism of protein.

Ackermann and his associates (1954) reported their observations of the calcium and nitrogen balance in 6 elderly women before and during sufficient estrogen therapy to reinstate menstruation. Their results indicated that estrogen therapy had no appreciable influence in those individuals who were in positive nitrogen and calcium balance. There was, however, a slightly increased retention of calcium and nitrogen in those showing an initial negative balance. The administration of progesterone also had only slight effect but testosterone produced a marked increase in nitrogen and calcium retention.

The administration of androstanolone to subjects 55 to 65 years of age in doses as low as 25 mg a day produced an increased nitrogen retention without affecting the urinary excretion of sodium chloride potassium or creatinine according to Pearson *et al* (1954). Metabolism balance studies by Watkin and his associates (1955) in 8 men 70 to 92 years of age also indicated that stanolone caused an increased nitrogen potassium and phosphorus retention which was greater than that produced by a high protein diet alone. The quantitative results however were greater when the two were combined. The subjects showed a significant increase in the urinary excretion of 17 ketosteroids.

It has been known for some time that the androgens possess the ability to promote protein anabolism thereby enhancing tissue synthesis. The clinician however has been hampered in their use by the undesirable androgenic side effects (Drill 1958). Because of this efforts have been expended to develop a drug separating the androgenic and the anabolic activities of these steroids. Out of these efforts came the development of Nilevar<sup>3</sup> (17 $\alpha$  ethyl 17 hydroxy 19 nor-4 androsten-3 one), a new synthetic analog of the steroid group of testosterone progesterone cortisone aldosterone, and estradiol all of which function as hormones. Testosterone for instance in addition to its androgenic properties exerts an anabolic effect upon protein metabolism (Kochakian 1946). Nilevar is structurally intermediate between testosterone and estradiol and func-

<sup>3</sup> Brand of Norethandrolone (C. D. Searle and Co.)



7 adults recuperating from a period of medical or surgical stress. In Table IV we note that 5 subjects showed evidence of increased nitrogen retention in terms of changes in total nitrogen and urea output in the urine, suggesting a reduction of tissue catabolism. In 6 subjects the urinary excretion of amino nitrogen increased. It is also evident that there was a net increase in the total excretion of the 7 amino acids measured chemically with methionine, phenylalanine, and tryptophan accounting for 95% of the increase and methionine alone contributing up to 50% of the total net increase. The metabolic significance of this definite aminociduria in the face of the reduced catabolism associated with the administration of the androgen remains to be explored.

Table IV also indicates the net changes in the blood proteins, hemoglobin, hematocrit, and body weight for each subject. Because of the short duration of the test periods it is not surprising to find poor correlation between nitrogen retention and changes in these factors. The 2 subjects (No. 5 and 7) showing the greatest increase in weight were likewise the only subjects showing an increased urinary excretion of total nitrogen. This presents the anomaly of a negative nitrogen balance with a positive weight gain not supported by a change in the hematocrit which would indicate any significant effect upon the water balance.

Kountz (1951) reported long range investigations of a group of women 50 to 95 years of age. He divided the patients into a control group and a test group who received 1 mg of estradiol twice a week with the addition of 5 mg of progesterone or testosterone daily for varying periods of time. He produced evidence that the sex hormones revitalized certain tissues particularly those of the genital tract which were restored to an appearance and function compatible with a younger age group. He also reported that there appeared to be a slowing down of the mental and physical decline often experienced by oldsters.

A review article by Odendaal (1952) discussed endocrine and nutritional considerations in the aged. He stated that the normal equilibrium between the anabolic effect of the 17 ketosteroids and the catabolic effect of the 11 corticosteroids tends to be upset by the aging process with the catabolic effect becoming predominant. He felt that the metabolic equilibrium might be protected by optimal nutrition which demands more of the essential nutrients than is commonly believed necessary for the old age group.

The consensus of opinion arising from a symposium regarding the relationship between the endocrines and the nutritional status in the aged was reported by Davidson (1954). It was suggested that endogenous endocrines having an anabolic effect upon protein synthesis show a gradual decrease in function with progressive aging which may alter

dosage did not indicate any reduction of insulin function of the pancreas.

Wisotsky and his associates (1945) substantiated the above findings with regard to the type of glucose tolerance curve found in oldsters. Their studies of aged males summarized in Fig 2 indicate a decreased tolerance for orally administered glucose. The blood sugar curve following intravenous glucose injections as reported by Smith (1950), also required a longer time to return to the normal pre injection level in oldsters as compared with young adults. The blood pyruvate, however, rose normally in the aged subjects which is contrary to the finding in

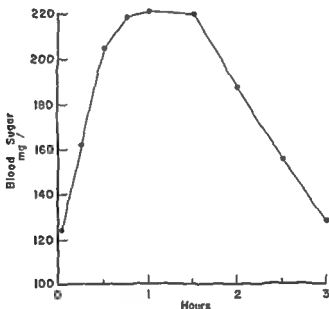


FIG 2 Mean blood sugar levels in a group of 14 males 60 to 70 years of age before and after an average oral test dose of 50 gm of glucose. Adapted from Wisotsky *et al* (1945)

diabetics. He concluded that the delayed type of glucose tolerance curve in the oldster was not due to insulin deficiency.

Further studies on the intravenous glucose tolerance test in different age groups were reported by Schneeberg and Finestone (1952) who found a slower response in subjects over 40 years of age. They believed, however, that this was not caused by liver dysfunction, deficient stores of oxygen, or a previously low carbohydrate intake, and that the normal oldster can probably handle glucose as well as the younger age group. Chesrow and Blever (1954) studied 46 men and 34 women from 60 to 109 years of age. Oral glucose tolerance tests were carried out on all subjects, and those showing prolonged curves were then subjected to an intravenous glucose tolerance test 8 days later. There was no uniform relation of the tolerance curve to the age of the individual. Intravenous

tionally preserves the anabolic effect of testosterone upon protein metabolism and tissue synthesis in rats while showing a marked reduction in the androgenic effect (androgenic anabolic ratio of 1:16 compared to testosterone propionate) (Drill and Saunders 1956). Further observations of rats indicated that Nilevir produces an increased nitrogen retention without significant change in sodium potassium or calcium retention. It does not possess cortisone like activity, does not show estrogenic effects, is of a low order of toxicity and is effective when administered parenterally or orally.

Albanese and his group (1958a) working with a very similar compound 19 nortestosterone (17 $\alpha$  17 hydroxy 19 nor-4 androsten 3 one) studied its effect on elderly male patients who had not shown a positive nutritional response to an adequate well balanced diet. A group of convalescent men 54 to 85 years of age received the normal convalescent hospital diet for the first week, the same diet plus 75 mg of 19 nortestosterone orally during the second week, the same diet plus 750 mg of L lysine and 75 mg of 19 nortestosterone daily, for the third week and the same diet plus only 750 mg of L lysine daily, for the fourth week. Nitrogen balance, urinary creatinine, serum sodium, potassium, calcium and cholesterol were determined for each period. The results may be summarized as follows: (a) The nitrogen retention and the body weight showed the greatest increments during the third week in which the regular diet was supplemented by L lysine and 19 nortestosterone. (b) There was also some increase in nitrogen retention during the second week period on diet plus 19 nortestosterone alone. (c) 19 Nortestosterone had no appreciable effect upon blood sodium, potassium or calcium levels. (d) The effect upon blood cholesterol levels was inconsistent and not statistically significant.

#### IV CARBOHYDRATE METABOLISM IN THE AGED AND ITS RELATION TO PROTEIN METABOLISM

The metabolism of carbohydrates in the aged has been studied by many workers during the past twenty years. We will attempt to summarize the more essential reports particularly those related to protein nutrition.

Bogdanovich (1940) concluded that the fasting blood sugar was of the same magnitude in the older and the young adult. The amount of glycogen in the blood was definitely increased in the older age group suggesting that glycogen fixation in the liver was diminished. The blood sugar curve after an oral dose of 50 gm of glucose showed a slower rise and a more prolonged elevation of the blood sugar in the elderly which however, reached normal peak values. Experiments with double glucose

dosage did not indicate any reduction of insulin function of the pancreas

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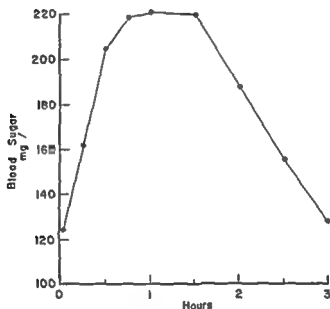


FIG 2 Mean blood sugar levels in a group of 14 males 60 to 70 years of age before and after an average oral test dose of 50 gm of glucose. Adapted from Wisotsky *et al* (1945)

diabetics. He concluded that the delayed type of glucose tolerance curve in the oldster was not due to insulin deficiency.

Further studies on the intravenous glucose tolerance test in different age groups were reported by Schneeberg and Finestone (1952) who found a slower response in subjects over 40 years of age. They believed, however, that this was not caused by liver dysfunction, deficient stores of oxygen, or a previously low carbohydrate intake, and that the normal oldster can probably handle glucose as well as the younger age group. Chesrow and Bleyer (1954) studied 46 men and 34 women from 60 to 109 years of age. Oral glucose tolerance tests were carried out on all subjects, and those showing prolonged curves were then subjected to an intravenous glucose tolerance test 8 days later. There was no uniform relation of the tolerance curve to the age of the individual. Intravenous

glucose tolerance tests were also carried out on 16 subjects showing prolonged curves after oral glucose, and none of these subjects showed a diabetic type curve

Between 1951 and 1955, Albanese and his group reported the results of various experiments concerning the utilization of carbohydrates and their effect upon protein metabolism. We will attempt to record here only a brief summary of their findings

Observations on the parenteral use of invert sugar (Albanese *et al*, 1951a) indicated the need for a critical evaluation of the protein sparing action of various carbohydrates. Their data indicated that nitrogen retention and utilization was greatest with diets in which 70% of the total carbohydrate fraction was fructose. They also reported (Albanese *et al*, 1952b) data to substantiate the fact that invert sugar infusions have certain advantages

1 The rapid utilization of the fructose portion provides a readier source of energy than does glucose

2 Fructose has a greater protein sparing effect than glucose, at adequate levels of nitrogen intake

3 This particular property is of considerable value in reducing the undesirable nitrogen loss which accompanies any trauma

4 These advantages are secured without hyperglycemia. Therefore the subsequent hypoglycemic rebound and the diuresis which accompanies glucose infusions are avoided, suggesting that fructose is a better sugar for parenteral carbohydrate administration

Further experience by Albanese and his group (1954a) showed that at the 50 and 100 gm levels of administration, intravenous fructose solutions resulted in a greater nitrogen retention than invert sugar solutions, which in turn showed greater protein sparing effect than glucose. They concluded that 5% fructose solution or 10% invert sugar solution appear to be the nutrients of choice for intravenous alimentation

The importance of the protein sparing action of carbohydrates in the treatment of cirrhotic patients led to the following investigations which have not been published to date (Albanese *et al*, 1956). A group of 16 postacute respiratory disease patients with normal liver function tests, and another group of 10 patients with definite clinical and laboratory evidence of active cirrhotic disease were both studied with regard to the changes in plasma amino nitrogen levels during and after the intravenous administration of fructose or glucose. The change was expressed as an index (plasma amino nitrogen index), determined as follows

$$\text{Index} = \frac{\text{Maximal plasma amino N level} - \text{Fasting plasma amino N level}}{\text{Fasting plasma amino N level}} \times 100$$

Each individual in both groups received two intravenous infusions, one of glucose and one of fructose containing 0.5 gm of the test sugar per kilogram of body weight in a 10% solution administered over a 30-minute period. Fasting blood specimens showed no significant differences in sugar or amino nitrogen levels between the two groups. The amino nitrogen level was redetermined every 15 minutes for 1 hour after the infusion started and the sugar levels were measured after 30 and 60 minutes. The mean plasma amino nitrogen index was definitely greater with the fructose infusion versus the glucose infusion in both the normal and the cirrhotic groups as indicated in Table V. This would suggest a greater and more rapid conversion of fructose to amino acids thereby

TABLE V  
AMINO NITROGEN INDEX CHANGES FOLLOWING INTRAVENOUSLY ADMINISTERED  
GLUCOSE AND FRUCTOSE

Subjects	Glucose	Fructose	P
Normal (13-67 years)			
Number of patients	13	16	
Index	5	20	0.1
Cirrhotic (32-80 years)			
Number of patients	9	10	
Index	9	19	0.5

enhancing its protein sparing action over that of glucose. It was also noted that the mean blood sugar levels following the infusions were definitely lower when using fructose as compared to glucose in both patient groups and the mean blood sugar levels at various time intervals were essentially the same in both the normal and the cirrhotic groups.

The same investigators (Albanese *et al.* 1954b) studied the effect of age on the utilization of various carbohydrates administered orally. The utilization of glucose decreased markedly with age whereas the utilization of fructose was only slightly affected. Invert sugar and sucrose were utilized better than dextrose but not as well as fructose alone. Lactose was utilized better than dextrose but less so than sucrose, invert sugar, or fructose in the aged. The elderly insulin controlled diabetic also showed a better utilization of fructose than dextrose. These workers believed that fructose or fructose containing sucrose are the sugars of choice for the aged.

Investigation of the effect of carbohydrates upon the metabolic nitrogen pool (Albanese *et al.* 1954c) disclosed that the more readily utilized fructose makes an appreciable positive contribution to the metabolic nitrogen pool within 1 hour which is not true for dextrose as shown in Table VI. In feeding experiments (Albanese *et al.* 1955c) subjects from

glucose tolerance tests were also carried out on 16 subjects showing prolonged curves after oral glucose, and none of these subjects showed a diabetic type curve

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5 to 81 years of age were fasted for 12 hours overnight. Oral test doses of different quantities of a protein preparation (0.04 gm per kilogram), or different carbohydrates (10 gm per kilogram) were administered dissolved or suspended in 250 ml of water. Blood samples were collected before and again exactly 1 hour after the test dose. Total blood sugar and fructose concentrations were determined colorimetrically. Blood amino nitrogen levels were determined in deproteinized filtrates. The results of this work again indicated that fructose is more rapidly metabolized than glucose and results in the rapid formation of carbon structures which by combination with the labile amino groups become available by the transamination cycle for conversion into amino acids.

TABLE VI  
EFFECT OF ORAL ADMINISTRATION OF VARIOUS CARBOHYDRATES ON THE METABOLIC NITROGEN POOL

Test sugar	Differences from fasting levels mg %		
	Amino nitrogen	Total sugars	Fructose
Fructose	1.02	24.3	4.9
Dextrose	-0.61	52.0	0.5
None	-0.47	-10.5	0.0
Fasting levels mg %	5.82	101.8	1.8

The protein sparing effect of carbohydrates and fats was studied by Kountz and his group (1955). Four men 70 to 89 years of age and 5 women 68 to 82 years of age, had been in positive nitrogen balance on a diet containing 1.1 gm of protein and 35 to 40 calories per kilogram per day. When 400 calories per day were added to an isocaloric diet in the form of carbohydrate or fats, a protein sparing effect equivalent to an extra 5 to 10 gm of dietary protein per day was estimated on the basis of nitrogen retention measurements.

The fallibility of the glucose tolerance test in the aged was questioned by Wagner (1955). He studied 106 women 65 to 85 years of age who showed no evidence of diabetes, liver disease, or abnormal thyroid function. The routine oral glucose tolerance test was administered at the beginning of the experiment, was repeated after 1 year in 82 subjects, and was repeated again at the end of the second year in 27 subjects. The results showed a wide variety of high prolonged curves such as those reported previously by Bogdanovich (1940), Wisotsky *et al* (1945), and Chesrow and Bleyer (1954). He substantiated the previously reported findings of others regarding the lack of correlation between a type of glucose tolerance curve suggestive of the diabetic and the clinical state of the elderly subject. He suggested that the most reliable

index of a true metabolic defect in the carbohydrate metabolism is a constant elevation of the fasting blood sugar level. Secondary importance is assigned to the failure of the curve to reach the pretest level in 3 to 4 hours. The least reliable index is the blood sugar at the peak of the curve.

## V BLOOD LIPIDS AND CHOLESTEROL AND THEIR RELATION TO PROTEIN METABOLISM

Twenty years ago the problems relating to blood cholesterol levels in the older age groups were being argued controversially. Pierret *et al* (1939) reported that in their experience they found no changes in blood chemistry which were statistically significant of old age and that the cholesterol level varied and showed no correlation with the nonprotein nitrogen in the blood or with the arterial blood pressure.

A few years later Rafsky and Newman (1942) carried out studies in a group of 100 normal elderly subjects. When whole blood levels were measured only 57% of the subjects showed a total cholesterol concentration greater than 200 mg % while 68% of the same group showed a free cholesterol level greater than 40 mg %. These findings were considered to be within the normal range. When 50% of the subjects, however, had determinations made upon the serum rather than the whole blood, 74% showed a total cholesterol concentration above 200 mg % while 54% showed a free cholesterol level greater than 40 mg %. (No explanation was offered for the variation between the whole blood group and the serum group.)

Foffani and Berti (1947) reported their findings regarding the relationship between the protein content of peripheral and medullary blood and hyperlipemia in an aged group 62 to 87 years of age as contrasted to young adults under 50 years of age. They found that the protein content of the peripheral blood of the oldsters averaged about 1 gm % lower than the young adult group. The index of refraction in the peripheral blood was in the range of 1.34717 to 1.35014 in the majority of the aged as contrasted to a range of 1.35014 to 1.35338 in the younger group. They also found that there was an increased hyperlipemia in the medullary blood as contrasted to the peripheral blood in the oldster. They felt that this factor explained some of the apparent variability of medullary protein values and of the A/G ratio.

Albanese (1952c) referred to previous experiments (Albanese *et al* 1951b) upon the output of nitrogenous metabolites following the intravenous infusion of 5% bovine plasma digest with and without 5% dextrose and/or ethanol. When a fat emulsion was administered orally along with the infusion, a further improvement in amino acid utilization was noted. Feeding experiments indicated that an orally administered milk

protein concentrate suspended in milk caused a positive nutritional response in terms of body weight change which was greater in the oldster than in the young adult. By adding oral fat emulsion to this protein supplement he noted further improvement in nutritional response. This was thought to be due to the presence of an increased number of energy calories with a subsequent increase in the protein sparing effect. Further experiences with this modality were reported by Higgons and Albanese (1957) as outlined in Section III, C, 1 of this Chapter.

The consensus of opinion of various investigators (Eiber *et al.* 1954, 1955, Rossi, 1954, Goldbloom, 1955, Ackermann *et al.* 1955) concerning serum lipids in the older age groups, can be summarized as follows. In subjects 80 to 100 years of age the average total serum lipid level was lower than that found in the normal or atherosclerotic younger subjects. On the other hand, there was no statistical difference in the serum total cholesterol or phospholipid level, between the two age groups. The phospholipid/total cholesterol ratio was less than 1.00. Standard  $S_1$  0-12 and  $S_1$  12-400 lipoprotein fractions and the atherogenic index all declined considerably with advanced age. Studies of the serum lipoprotein by paper electrophoresis showed no statistical differences between the 80 to 90 year group and the 30 year group. For the first time the correlation between paper electrophoretic and ultracentrifugal methods of serum lipid investigation in the aged was pointed out by Goldbloom (1955). He found no significant difference in the lipoprotein distribution in old age as compared with young adults. Investigation of 650 subjects ranging from 20 to 106 years of age (by various methods: chemical lipoprotein electrophoretic patterns of the blood, X-ray studies of the aorta, electrocardiogram, pathological changes in retinal vessels, pathological changes in aorta and coronary arteries) showed a tendency for total blood lipids, certain lipoproteins, the atherogenic index and the degrees of aortic calcification and dilatation all to rise gradually until the age of 60 to 75 years and thereafter to fall. There was also a slight decrease in the  $S_1$  12-20 level with age, and a good correlation between this  $S_1$  level and the cholesterol level. The phospholipid level varied linearly with the cholesterol level.

Gillum and co workers (1955) reported a positive correlation between the dietary intake of cholesterol and fat and the corresponding blood levels for these factors. There was also a slight positive correlation between the dietary protein intake and the cholesterol and lipid blood levels. In men who were 20% or more below or above their standard expected weight, the underweight group showed a low blood cholesterol while in the overweight group the reverse obtained. This finding did not apparently occur in women, or in men showing a deviation from standard weight of lesser magnitude than 20%.

A group of 29 healthy institutionalized women were studied by Walker *et al* (1956) regarding serum cholesterol levels in reference to age. The older group of 14 subjects had an average age of 64 years and the younger group of 15 subjects had an average age of 31 years. Calculation of the food intake throughout the 4 months of study indicated that the average diet contained at least 100% of the daily allowances recommended by the National Research Council (1953). A mean serum cholesterol level of  $230 \pm 9$  mg % was found in the older group and  $172 \pm 8$  mg % in the younger group. The correlation between age and serum cholesterol level was significant at the 1% level. Since diet calculations indicated a more or less identical food intake, these workers felt that the increased cholesterol in the older group was related to age rather than to diet.

Feeding experiments relating dietary factors to serum lipoprotein levels in 5 young to middle aged men were reported by Nichols and his group (1957). On isocaloric diets the standard  $S_1$  0-12 and  $S_1$  12-20 lipoproteins in the serum were greatly elevated when the diet was high in animal fat, as contrasted with a diet high in vegetable fat or low in total fat with high carbohydrate. They also reported an apparent dissociation between the effects of diet upon the  $S_1$  0-20 lipoproteins and the  $S_1$  20-400 lipoproteins. On certain diets both of these classes changed in the same direction while on other diets they might change in opposite directions. Since both classes are cholesterol bearers this dissociation could be obscured if the only measurement made was that of the serum cholesterol.

Jolliffe (1957) published a review article regarding fats and cholesterol in coronary heart disease. The article concluded as follows: The presence of certain marine and vegetable oils in the diet caused a fall in the  $\beta$  lipoproteins and total cholesterol levels in the blood. The less saturated the oils the greater the reduction in blood cholesterol. On the other hand if the dietary intake of saturated fats was increased the blood levels of cholesterol and lipoprotein also increase. This author went on to point out that statistical studies had shown significant differences in the occurrence rate of and death rate from coronary heart disease between various countries and various population groups in the same country. He stated that although environmental factors had been suggested the correlation had never been proven. He did feel however that there was a correlation between coronary disease and (a) the per cent of total dietary calories derived from fats (Keys and White 1956) (b) a change in dietary intake of hydrogenated and highly saturated fats both of which lead to an increase in the cholesterol level of the blood. It appeared that a deficient intake of unsaturated fatty acids leads to the

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Feeding experiments relating dietary factors to serum lipoprotein levels in 5 young to middle aged men were reported by Nichols and his group (1957). On isocaloric diets the standard S<sub>1</sub> 0-12 and S<sub>1</sub> 12-20 lipoproteins in the serum were greatly elevated when the diet was high in animal fat as contrasted with a diet high in vegetable fat or low in total fat with high carbohydrate. They also reported an apparent dissociation between the effects of diet upon the S<sub>1</sub> 0-20 lipoproteins and the S<sub>1</sub> 20-400 lipoproteins. On certain diets both of these classes changed in the same direction while on other diets they might change in opposite directions. Since both classes are cholesterol bearers, this dissociation could be obscured if the only measurement made was that of the serum cholesterol.

Jolliffe (1957) published a review article regarding fats and cholesterol in coronary heart disease. The article concluded as follows: The presence of certain marine and vegetable oils in the diet caused a fall in the  $\beta$  lipoproteins and total cholesterol levels in the blood. The less saturated the oils the greater the reduction in blood cholesterol. On the other hand if the dietary intake of saturated fats is increased the blood levels of cholesterol and lipoprotein also increase. This author went on to point out that statistical studies had shown significant differences in the occurrence rate of and death rate from coronary heart disease between various countries and various population groups in the same country. He stated that although environmental factors had been suggested, the correlation had never been proven. He did feel however that there is a correlation between coronary disease and (a) the per cent of total dietary calories derived from fats (Keys and White 1956) (b) a change in dietary intake of hydrogenated and highly saturated fats both of which lead to an increase in the cholesterol level of the blood. It appeared that a deficient intake of unsaturated fatty acids leads to the

formation of a saturated fatty acid cholesterol complex which is less soluble in the blood stream and therefore might be deposited more readily in the intima of high pressure arteries (Sinclair, 1956)

Albanese and Higgons with their group had been making observations in this field for some time. One of their reports (Albanese *et al* 1958b) stated some of the results of their studies of factors influencing total blood cholesterol levels in the geriatric field. The subjects studied came from three groups: residents of the Miriam Osborn Memorial Home for elderly women, older patients at St Luke's Convalescent Hospital and volunteers from the lay staffs of both institutions. Periodic analyses of food intake were carried out, and indicated that on a self selected diet the elderly ladies at the Osborn Home (70 to 99 years of age) showed an average daily caloric intake of  $1560 \pm 197$  while the patient group at the Convalescent Hospital showed an average intake of  $2000 \pm 190$  calories. The distribution of these calories in both groups was approximately: protein, 14%, carbohydrate, 44% and fat, 42%. The elderly ladies showed an average daily intake of  $72 \pm 11$  gm of fat most of which was derived from meat dairy products, and hydrogenated oils (largely saturated fats). Repeated studies of the blood cholesterol levels in this group indicated that the average level was  $248 \pm 46$  mg % in the age group 70 to 79 years,  $229 \pm 32$  mg % in the age group 80 to 89 years and  $228 \pm 43$  mg % in the age group above 90 years. In other words, there was an apparent downward trend in the blood cholesterol levels starting in the sixth decade of life and continuing through the seventh eighth and ninth decades. The relationship of the blood cholesterol level to gross body weight showed no significant variation for individuals whose weight ranged from 70% to 130% of the standard expected.

Eight ambulatory elderly ladies (above 70 years) from the Osborn Home whose average fat intake was 72 gm per day and in whom the plasma cholesterol levels averaged 271 mg % were selected for the administration of an oral fat emulsion which yielded 27 gm of total fat per day, half of which were unsaturated fatty acids from soybean oil. It was found that about 40% of these subjects showed a decrease in blood cholesterol level and 60% showed an increase during the administration of the fat emulsion supplement.

Parallel studies of 4 male staff members (30 to 60 years of age) at the Home with an average pretest blood cholesterol level of 219 mg %, showed a significant decrease while on the fat emulsion supplement in the blood cholesterol level averaging  $-26$  mg % suggesting that this cause and effect factor is more effective in the younger adult male group than in an older female group.

Sixteen ambulatory patients at St. Luke's Convalescent Hospital (average age 62 years) with acknowledged chronic cardiovascular disease were given the same fat supplement for periods of 1 to 5 weeks (No pretest blood cholesterol levels were available.) The results varied considerably between individuals. In attempting to relate the maximum change in the blood cholesterol level with the age of the individuals we find that only 3 of the 9 patients showing an increased level were over 70 years of age. This may be related to the observation described above that individuals in the seventh to ninth decades show a tendency to a lower blood cholesterol level.

These investigators summarized their experience by stating that the results suggested (a) In the older a body weight above 100% of expected standard is not necessarily associated with blood cholesterol levels above 200 mg %. (b) Increasing age is often accompanied by blood cholesterol levels above 200 mg %. (c) Administration of a soy bean oil emulsion containing about 50% unsaturated fatty acids appeared to have an equalizing effect upon the blood cholesterol level i.e. those individuals with a pretest level above 200 mg % showed a significant decrease in the blood cholesterol during the fat emulsion feeding period whereas for those individuals with a pretest level below 200 mg % there was an increase in the blood cholesterol level during the fat supplement period.

In the above investigations the total plasma cholesterol was determined by a modification of the method described by Zlatkis *et al* (1953). The total plasma proteins A/G ratios and lipoproteins were determined by microtechniques previously reported by Albanese *et al* (1955).

More recently the same group of investigators (Albanese *et al* 1958c) reported further investigations of cholesterol metabolism and its relationship to protein and amino acid metabolism. They believe there is now ample experimental evidence that cholesterol metabolism by means of various enzyme systems may be affected by dietary fats, carbohydrates or proteins. It appears that dietary fats with a high degree of saturation tend to produce higher blood cholesterol levels than do the unsaturated vegetable oils (Kinsell *et al* 1952 Ahrens *et al* 1957 Albanese *et al* 1958b). It also appears that fat formed from carbohydrates is low in polyunsaturated fatty acids (Nichols *et al* 1957). Fructose appears to be less lipogenic than glucose (Albanese *et al* 1955d). The effect of dietary proteins upon the blood lipids is still under discussion. It is believed that proteins when ingested in large amounts may be used for the synthesis of saturated fats. This effect may be counterbalanced by certain amino acids such as methionine and threonine which exert a lipotropic effect (Okey and Lyman 1956).

Albanese and his associates (1958c) further investigated the effects of an increased protein intake upon the blood cholesterol levels of 16 men and women with an average age of 60 years and with an average weight about 80% of the expected standard (Metropolitan Life Insurance Company, 1951). Analysis of their diet showed an average intake of 65 gm of protein (0.5–0.7 gm per pound) and 2000 calories per day. The pretest plasma cholesterol levels for the whole group averaged 220 mg %. A dietary supplement of Somagen\* 45 gm suspended in 500 ml of water, divided into three portions, was fed between meals and at bed time. This supplement added about 30 gm of milk protein and 180 calories to the daily diet and increased the dietary protein calories from a pretest level of 13% to a test period level of 18% of the total daily caloric intake. The supplement was continued for an average period of 19 days. Three individuals with a pretest average blood cholesterol level of 221 mg % showed a decrease in blood cholesterol level averaging  $-14$  mg %, one individual with a pretest cholesterol level of 189 mg % showed no change, the remaining 12 subjects whose average pretest cholesterol level was 224 mg % showed an increase in plasma cholesterol averaging  $+28$  mg %. These data suggest a lipogenic effect from the high protein supplement with some of the protein being converted to cholesterol.

Since the effect of dietary protein upon the blood lipids is usually ascribed to the labile methyl group coming from the methionine content, and since choline and betaine have been shown by other investigators (Du Vigneaud 1948, Blumberg *et al.*, 1956) to be an equally good source of methyl as a hypotrophic agent, our group studied the effect of a methylating agent<sup>5</sup> upon the blood cholesterol level. A group of 11 men and women who were patients in a high grade convalescent home (Burke Foundation) were selected for study. The group showed an average age of 65 years, average weight about 90% of expected standard, average daily intake of about 1960 calories with fat calories averaging 43% of the total and protein calories averaging 16% of the total caloric intake. The pretest blood cholesterol levels for the whole group averaged about 200 mg %. Each patient was given 5 ml of the methylating agent 3 times a day with meals for an average period of 37 days. Five individuals with an average pretest cholesterol level of 197 mg % showed a decrease in the blood cholesterol averaging  $-18$  mg %, the remaining 6 individuals with an average pretest cholesterol level of 212 mg % showed an increase in blood cholesterol averaging  $+13$  mg % while taking the

\* The Upjohn Company, Kalamazoo, Michigan.

<sup>5</sup> Smith, Kline and French Laboratories, No. X77J. Each 5 ml contains betaine 700 mg, pyridoxine 20 mg, nicotinamide 70 mg, and vitamin B<sub>12</sub> 8.3 µg.

methylating agent. This and other evidence (Blumberg *et al.*, 1956) suggests albeit inconclusively that betaine may be an effective lipotropic agent for some individuals under these dietary conditions.

## VI GENERAL DISCUSSION

The literature is replete with references of a general nature regarding various opinions concerning the proper nutrition of oldsters. Many of these opinions are open to criticism due to the lack of factual data, to the small numbers of subjects studied, or the subsequent development of different results through the use of more accurate modalities of study.

In the decade from 1940 to 1950 many observers (Tuohy, 1943; Spies and Collins, 1946; Pyle *et al.*, 1947; Ohlson *et al.*, 1948, 1950) stressed the importance of a balanced diet for the oldster, with a protein intake of about 1 gm. per kilogram per day (10 to 15% of the total calories), and a gradually decreasing essential intake of total calories with advancing age. Most workers felt that the intake of vitamins and minerals would be adequate if a proper diet were consumed. Reference was made regarding the effect of dietary habits upon the nutrition of the elderly. It was found that a group of oldsters who were in apparent good health were in the habit of consuming more milk, citrus fruits, whole grain cereals, vegetables, and eggs—as contrasted with a similar age group having “poor” health (Ohlson *et al.*, 1948).

Albanesc (1950a) pointed out that the availability and intake of protein foods are of vital importance since the mammalian body is unable to manufacture the essential nutrient protein without the dietary intake of all the essential amino acids needed for the formation of tissue proteins, enzymes, vitamins, and hormones. He also stressed the importance of the amino acid pattern of the dietary proteins, which determines their biological usefulness for man. Ten of the 23 amino acids occurring in proteins cannot be produced in the body and must be present in the diet in sufficient quantities and at the proper times to enable the human to synthesize his own tissue proteins. It is believed that the presence of the remaining 13 amino acids is also important, as they appear to have a sparing effect upon the quantities of essential amino acids necessary for tissue synthesis.

Albanese (1950b) stressed the point that pertinent data with the desired degree of accuracy were still lacking with regard to amino acid needs of the human adult, as illustrated by the divergences illustrated in Table VII. An increasing knowledge regarding the proper amino acid pattern should make it possible to recommend valuable supplementation of incomplete protein foodstuffs. Wheat proteins, for example, are known

to be poor in lysine but this deficiency could be corrected by the addition of milk which contains lysine

As long ago as the beginning of the century it was demonstrated by such investigators as Landergren (1903) Cathcart (1907) and Folin (1905) that an adequate supply of carbohydrate in the diet exerted a protein sparing effect upon the metabolism of man. A similar protein sparing action by the dietary fats was noted by Lusk (1928). Schwimmer *et al* (1945-1946) demonstrated that a reduction in total caloric intake tended to impair nitrogen retention. Braunstein (1947) reported interrelationships between any one individual's nutritional status and the pro

TABLE VII  
COMPARISON OF SUGGESTED DAILY AMINO ACID NEEDS OF MAN

Amino acid	Rose <sup>a</sup> (gm /70 kg man)	Harte and Travers <sup>b</sup> (gm /70 kg man)	Harte vs Rose (% difference)
Isoleucine	0.70	1.20	+ 70
Leucine	1.10	1.70	+ 55
Lysine	0.80	0.80	0
Methionine	1.10	0.50	- 55
Phenylalanine	1.10	1.40	+ 27
Threonine	0.50	1.00	+100
Tryptophan	0.25	0.40	+ 60
Valine	0.80	1.10	+ 38

<sup>a</sup> See Rose (1949)

<sup>b</sup> See Harte and Travers (1947)

tein metabolism and activity of regulatory agents such as hormones, enzymes and vitamins. Albanese felt that increasing discoveries of previously unknown essential or supportive nutrients made it advisable to carry out feeding experiments designed to determine the requirement of any single nutrient against a background of an otherwise natural balanced diet. This would allow for the normal protein sparing effects, essential amino acid sparing, the proper distribution of the specific dynamic action, and the biocatalyst amino acid interrelationships as mentioned above.

Considerable interest in the metabolism of fats and cholesterol as well as their relationship to pathology of the vascular system has been shown by various investigators. Albanese (1953) referred to studies carried out by Bloch (1945) which indicated that the human body could synthesize cholesterol from amino acids derived from the dietary proteins. Keys and his co-workers (1950) expressed doubt as to whether a significantly high blood cholesterol level could be induced in man by diet alone. Moon and Run (1952) offered evidence indicating that protein rather than fat is the cause of atherosclerosis, a degenerative

pathology starting with a change in the normal protein pattern of the blood vessel wall. Hegsted and his associates (1952) reported that the rate of cholesterol synthesis by the body increases as the dietary lipid intake decreases. Albinese (1953) felt that from the data available it would appear unnecessary to radically restrict the dietary fat intake in the aged. In subjects over 65 years of age special diets being used for any reason should be constructed in such a manner that the biochemical balance of the essential nutrients is not radically disturbed.

The quality of the dietary protein was discussed by Albinese (1954). He pointed out that the biological value of a dietary protein is based upon the percentage of the nitrogen intake retained by the body. In animals this may be estimated by means of (a) bio assay methods in young animals, (b) test diets after a control period in adult animals, and (c) repletion experiments in adult animals following a period on protein free diet with changes in body weight as the yardstick. Results indicate that a protein of low biological value (deficient in one or more amino acids) will cause a weight loss more rapid than that induced by a protein free diet. By adding a whole protein with a known adequate amino acid balance or by adding the deficient amino acid itself the weight curve is reversed.

In addition to the above techniques dietary experiments in man with regard to the nitrogen balance, total plasma proteins, blood hemoglobin and amino acid levels in the blood and urine are being used in an effort to evaluate the protein content of the diet. Unfortunately in man the results of these tests often do not correlate accurately with the nutritional status of the individual. It is also true that the biological value of a given nutrient varies with the particular amino acid needs at a particular time for the synthesis of a particular tissue.

In man the method of assigning a biological value to a dietary protein by measuring its amino acid pattern is now being used. The amino acid pattern of the test food which most closely approximates the pattern of the human tissues being synthesized is assigned the highest nutritional value. Unfortunately errors in technique of amino acid analysis still handicap this method.

Shea and her associates (1954) reviewed the protein requirements of the aged and found that a large percentage of elderly women on self selected diets were in negative nitrogen balance in spite of a dietary intake of protein which appeared adequate according to existing standards. They felt this was due to a gradually decreasing intake of proteins of animal origin by the older with substitution of more cereal proteins of lower biological value. They suggested the addition of essential amino acids to the cheaper cereal proteins to obtain a better amino acid pattern.



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The role of nutrition in the rehabilitation of the oldster, following illness, was discussed by Zintel (1955), Ferderber (1955), and Chinn (1956). They felt that the indications for nutritional therapy were the same for the oldster as for the young adult, and advised a well balanced relatively high protein diet as a means of reversing certain pathology of old age.

Martin (1956) discussed the place of nutrition in preventive geriatrics. He felt that degenerative disease could probably be controlled to some extent through preventive measures based upon sound principles of nutrition. There are, however, certain impediments to achieving this end: (a) Two thirds of the top soil in this country is deficient in minerals and organic matter, thereby producing foods of low biological quality. (b) The refinement and processing of foods tend to reduce their essential vitamin and mineral content. (c) Certain foods may at times contain small amounts of potentially toxic chemicals (insecticides). (d) Dyes used in food processing might be carcinogenic. (e) The administration of antibiotics, hormones, etc. to the animals to be used for human consumption, and the fluoridation of city water supplies—might eventually be detrimental to man.

Stare (1956) also felt that sound nutrition for the oldster is more or less identical with that for the young adult with the following differences: (a) Fewer calories are required, but the caloric intake should still be based upon the individual's activity. (b) There should be a larger proportion of protein calories in the diet at the expense of the fat calories. He suggested that future research might indicate the wisdom of a readjustment of the lipid intake so that a greater portion of the dietary fat calories are derived from the unsaturated fats. This change might offer more protection of the vascular system during advancing age than has been achieved by reduction of the total fat intake.

## VII SUMMARY

It is very difficult to summarize briefly this review type chapter. The text represents rather sharply condensed reports from many investigators. The ever increasing percentage of individuals past 60 years of age, and the forecast of an acceleration of this rate of increase in the coming years, serve to stimulate the interest of the nutritionist and the clinician for further studies in the basic nutritional needs of the oldster.

As long as 20 years ago, chemical analyses of human tissues were reported as showing changes in the electrolytes in the old versus the young age groups. Later work indicated that the oldster showed a fall in the specific gravity of the tissues, replacement of some active tissues by fatty tissues with a decrease in the water content. This tends to have an

adverse effect upon the accuracy of the standard height weight tables for the old age groups. Many workers reported the observation that the BMR decreases with advancing age but some felt that this decrease is more apparent than real and tends to disappear when the oxygen consumption is calculated in terms of lean body mass or unit of body water.

Slight functional impairment of the liver, kidneys and gastrointestinal tract was noted with increasing age but the majority of investigators felt that the normally healthy oldster shows little if any decrease in his ability to digest, absorb and utilize dietary nutrients.

Animal studies designed to correlate the diet and nutritional state with longevity indicated that the animals maintained on a balanced low calorie intake lived longer but enjoyed life much less than their litter mates who were maintained on unrestricted diets. Various testing modalities have been used in an effort to determine the optimal intake of protein (as well as other nutrients) in the old age group. The changing opinion through the years is reflected in the published recommended daily allowances of the Food and Nutrition Board, National Research Council (1945, 1948, 1953, 1958). The 1945 publication called for about 1 gm. of protein and 36 calories per kilogram per day for sedentary men with no allowance for increasing age. In contrast the 1958 revision recommends allowances for adults in the 25 year, 45 year and 65 year age groups. It calls for about 1 gm. of protein per kilogram of body weight per day for adult men and women in all 3 age groups. The recommended caloric intake for men however falls from 45 calories per kilogram per day for the 25 year old to 36 calories per kilogram for the 65 year-old and for women it falls from 40 calories per kilogram in the 25 year-old to 31 calories per kilogram per day for the 65 year old.

The relationship between protein intake and nitrogen balance has been widely investigated by many observers. Opinions regarding the fallibility of this modality vary considerably. Most nutritionists believe that the dietary intake of nutrients needed to maintain nitrogen equilibrium changes from time to time under the influence of such factors as mobility, physical or emotional stress, nutritional status and the biological value of the proteins being ingested.

It appears that the total plasma proteins tend to decrease with age and the A/G ratio falls slightly, usually at the expense of the albumin fraction. Increasing the dietary protein intake by the use of protein supplements does not as a rule have any profound effect upon the circulating blood proteins. Feeding experiments however indicate that caloric balance of the diet is of great importance to the proper utilization of nutrients. In an optimal diet for rehabilitation and repair of the undernourished oldster's tissues a caloric distribution of protein 15% car











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